Mathematical modeling of the early differentiation of helper T cells

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Modélisation mathématique de la différenciation précoce des lymphocytes T auxiliaires.

Soutenue le **20/02/2017** devant le jury composé de

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Résumé (Français) :

Les Lymphocytes T auxiliaires sont des acteurs essentiels de la réponse immunitaire spécifique contre des agents pathogènes, notamment par la production de cytokines adaptées. La différenciation des cellules T naïves $CD4^+$ en différentes populations effectrices (Th1, Th2, iTreg, Th17, ...) est contrôlée par le micro-environnement cytokinique. Toutefois, des études récentes ont montré que la composition en nutriments de l'environnement gouvernait le processus de différenciation des lymphocytes $CD4^+$. Dans cette étude, l'activation des cellules naïves $CD4^+$ en conditions limitantes en glutamine induit leur différenciation en lymphocytes T régulateurs Foxp3+ (Tregs). De plus, la conversion des cellules naïves en cellules Foxp3+ est également observée en condition de polarisation en Th1, corrélant aussi avec un blocage de la différentiation en cellules Th1.

Une approche de modélisation mathématique a été choisie, consistant d'équation différentielles, et conçue pour capturer les propriétés de différenciation des cellules T CD4 effectrices, dans un premier temps en conditions non limitantes en nutriments. Pour la phase d'apprentissage du modèle, les cinétiques d'expression des principaux facteurs de transcription et cytokines ont été mesurées *in vitro* dans les différentes conditions de différenciation. Ces données ont décelé des retards majeurs en terme de transcription, traduction et de sécrétion des cytokines, qui à leur tour façonnent l'enchaînement des boucles de rétroaction et décident de l'issue de la différenciation. Le modèle a reproduit avec succès la dynamique des différenciations 'canoniques', montrant que celles-ci peuvent être expliquées par un réseau de régulation relativement simple. Cependant, le modèle n'a reproduit qu'une partie des propriétés de plasticité des lymphocytes T CD4, et a besoin d'être affiné. Le modèle va être utilisé pour mieux récapituler la plasticité des lymphocytes T et comparer différentes hypothèses mécanistiques concernant l'impact de la glutamine sur la différenciation.

Mots-clés : système immunitaire; modélisation mathématique; lymphocytes T; différentiation; métabolisme.

Abstract (English):

T helper cells are essential actors of the immune response against specific pathogens notably through the production of adapted cytokines. The differentiation of naïve $CD4^+$ T cells into distinct effector subsets (Th1, Th2, iTreg, Th17, …) is regulated by the cytokine microenvironment. Notably though, recent studies have revealed a critical role of the nutrient environment in governing the terminal differentiation of $CD4^+$ T cells. In this work, activation of naïve $CD4^+$ T cells under conditions of glutamine deprivation resulted in their differentiation to $Foxp3^+$ regulatory T cells (Tregs). Moreover, the skewing of glutamine-deprived naive $CD4^+$ T cells into a $Foxp3^+$ fate occurred even under Th1 polarizing conditions, and correlated with a blocking of the Th1 effector fate.

A mathematical modelling approach using Ordinary Differential Equations was chosen to capture the properties of T cell effector differentiation, first in full nutrient conditions. In order to train the model, the kinetics of expression of master transcription factors and cytokines were monitored under distinct T cell polarizing conditions. *In vitro* data revealed major latencies in transcription, translation and cytokine secretion, which were important in deciding the timing of cytokine feedbacks and transcription factors expression. Our model successfully reproduced the dynamics of differentiation. These data confirm that 'canonical' *in vitro* differentiation can be explained by a simple regulatory network. Several, but not all early properties of plasticity of differentiating cells could be predicted by the model. Further modeling will be utilized to better account for T cell plasticity and compare mechanistic hypotheses linking glutamine sensing to differentiation.

(< 1700 characters)

Keywords: immunity; mathematical modeling; T cells; differentiation; metabolism.

Zusammenfassung (Deutsch):

T-Helferzellen sind erforderlich, angepasst an Infektionen definierte Zytokinantworten zu mediieren. Zahlreiche Untergruppen von T-Helferzellen mit spezifischen Funktionalitäten, wie zum Beispiel proinflammatorische Th1, Th2, Th17; und anti-inflammatorische Foxp3+ iTreg Zellen wurden bereits identifiziert. In der hier präsentierten Studie wurden die determinierenden Faktoren für die Differenzierungsentscheidung von naiven T Zellen untersucht.

Es konnte bereits gezeigt werden das essentielle Nährkomponenten Auswirkungen auf die Differenzierung T Zellen haben, wobei jedoch die Einfluss Glutamin bisher nicht untersucht wurde. In dieser Studie resultierte der Entzug von Glutamin in einer der Expression von Foxp3 unter Th1-Differenzierungsbedingungen. Darüber hinaus wurde die Th1 und Th17 Differenzierung beeinträchtigt. Diese Daten implizieren, dass in Glutamin-Armen metabolischen Umgebungen, wie z.B. soliden Tumoren, tumorunterstützende anti-entzündliche Reaktion unterstützt würden.

Auf diesen Ergebnissen aufbauend sollte eruiert werden, welche Auswirkungen, Glutamin auf regulatorischen Netzwerkentscheidend für die T-Helfer Differenzierung, hat. Eine mathematische Modellierung unter der Verwendung gewöhnlicher Differentialgleichungen, wurde gewählt um die Eigenschaften von T-Zell-Differenzierung, mit Glutamin, zu erfassen. Um das Modell zu trainieren, wurde eine Kinetik der Master-Transkriptionsfaktoren und Zytokineexpression über die Differenzierung von naiven T-Zellen in verschiedenen polarisierenden Bedingungen erstellt. Mittels der *in vitro* Daten konnte eine erhebliche Verzögerungen bei der Transkription, Translation und Sekretion von Zytokinen und der entscheidenden Differenzierungszeitpunkten bestimmt werden. Das mathematische Modell ermöglichte es, die Dynamik der Differenzierung in erfolgreich zu reproduzieren, und unterstreicht, dass die *in vitro* "kanonischen" Differenzierungen durch einfache regulatorisches Netzwerke erläutert werden können. Das plastische Verhalten von T-Zellen konnte nur zum Teil reproduziert werden. In zukünftigen Studien wird das Modell verwendet, um verschiedene mechanistische Hypothesen der T Zell-Differenzierung zu vergleichen, und zu bestimmen inwieweit Glutamin Einfluss auf die Differenzierung ausübt.

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ABBREVIATIONS:

AICD	Activation-Induced Cell Death
APC	Antigen Presenting Cell
ATP	Adenosine TriPhosphate
BCR	B-Cell Receptor
CAR	Chimeric Antigen Receptor
CRISPR	Clustered Regularly Interspace Short Palindromic Repeats
CTV	Cell-Trace Violet
EDTA	EthyleneDiamineTetraAcetic acid
FCS	Fetal Calf Serum
Foxp3 (*foxp3*)	Forkhead box P3 protein / gene
Gata3 (*gata3*)	GATA binding protein 3, (two isoforms) / gene
IFN-γ	Interferon Gamma
IMDM	Iscove's modified Dulbecco's medium
iTreg	induced Regulatory T cell
JAK	Janus Tyrosine Kinase (previously called Just Another Kinase)
MACS	Magnetic Activated Cell Sorting
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
mRNA	messenger RiboNucleic Acid
nTreg / tTreg	natural / thymic Regulatory T cell
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PRR	Pathogen Recognition Receptor
Rorγt (*rorc*)	Retinoic Acid Related Orphan Receptor gamma, shorter isoform / gene
RPMI	Roswell Park Memorial Institute medium
RT-qPCR	Reverse Transcription Polymerase Chain Reaction
SMAD	Single Mothers Against Decapentoplegic
STAT	Signal Transducer and Activator of Transcription
T-bet (*tbx21*)	T-box 21 protein / gene
TCA cycle	TriCarboxylic Acid cycle (Krebs' cycle)
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor β (implicitely, TGFβ1)

CHAPTER 1
INTRODUCTION

1.1 Main functions of the immune system

Broad definition of the immune system The immune system can be introduced as the set of cells and mechanisms that allow the body to react against pathogens and, generally speaking, any kind of body that can generate sickness, from microparticles to living organisms or tumors. Every cell of the body can actually be involved in an immune reaction, but the term 'immune cell' is commonly (and, in my opinion, quite unfairly) restricted to the cells that specialize in immune reactions. The immune system encompasses white blood cells (leukocytes), which recirculate among lymphoid organs through the blood; resident immune cells that scan tissues such as macrophages and dendritic cells; and the lymphoid organs (spleen, bone marrow, lymph nodes, tonsils, Peyer's patches).

Types of pathogen faced by the body At the scale of large multicellular eukaryotic organisms, the body is challenged by pathogens coming from all branches of the tree of life, namely:

- **Viruses:** these are inert molecular structures that carry their own genome and wait to insert it into a host-cell's genome to be able to duplicate their own genome. Since their genome is usually constrained by the small size of the capsid, they only encode a few specialized proteins and they need the host cell machinery of a living cell to produce and replicate new viral proteins. Some viruses infecting amoeba species harbor a very large size and genome [1] and own more genes that small living organisms, but they still require a living host to multiply. On the other hand, because they are inert, some viruses can persist in environments where alive organisms would die, like in ice. While

the infected cells usually die from the virus or from apoptosis as a protective reaction, some viruses can stably integrate their genome as DNA inside the chromosomes of the host cell, thereby subsisting in the cell and its progeny.

- **Bacteria:** these living organisms take advantage of a micro-environment with nutrients to proliferate. Their pathological properties are not directly linked to their presence or their nutrient consumption, but rather the virulence factors they express that challenge the body and can be toxic. Some bacteria are intracellular, hijacking the cell machinery and nutrients; others are extracellular and enter via contact with the body at mucosal barriers. As long as they are located outside the physical barriers of the body, they are harder to access by immune cells. The tricky aspect of bacteria is that they are ubiquitously present in our environment and especially along the mucosa and in the digestive tract. Some enteric bacteria are beneficial to the host by digesting complex nutrients that the human body does not process normally, such as polysaccharides [2], or by producing essential vitamins [3]. Germ-free rodents, thereby lacking gut microbiota, are less efficient in digestion than their colonized littermates, and eat substantially more calories [4]. The gut microbiota also competes with pathogenic bacteria for the colonization of the gut (reviewed in [5], and the disruption of a microbial community by antibiotics can sometimes open a window to opportunistic pathogens. The role of the immune system in the control of the microbiota, and especially the mechanisms allowing the immune system to tolerate commensals, and the mechanisms by which commensals control the immune system are a current field of study, and rely on multiple layers of defenses (reviewed in [6, 7]).

- **Eukaryotic cells**, monocellular or pluricellular: These can be fungi (candida albicans, causing mycoses for instance) or parasites such as worms or protists (ex: Leishmania). In a healthy state, their location is restricted to the 'outside' of the physical barriers of the body, meaning on the skin, the mucosa, in the gut and lung, and in the digestive tract, but not inside the body itself, which is supposedly kept sterile. They need to break physical barriers to invade the body and can have complex reactions against the defenses of the body.

Additional features of the immune system The functions of the immune system are not restricted to foreign pathogens but also include broader tasks that are necessary to maintain the body's homeostasis. An overall list of tasks and troublemakers cleared by the immune

system are shown in (Figure 1.1), including the clearance of dead cells, debris, tumors and pathogenic commensals. It is important to look at what the immune system tolerates, starting from cells and structures within the body. Some micro-organisms are additionally tolerated when they are outside the mucosa, while the same pathogens would be rejected inside the body. The distinction between which particles trigger an immune response and which ones are tolerated is crucial and still widely debated, and relies on complex layers of regulatory mechanisms in which helper T cell have a major impact.

1.2 Pathogen recognition and clearance mechanisms

In order to introduce the role of CD4 T cells, the immune system can be separated into several lines of defense that are modulated by them. I chose to present helper T cells being at the top of these layers because they regulate all other immune compartments through the production of cytokines. As each of these layers is evolutionarily conserved within different clades of species, I propose to start by looking at the immune defenses of other species and then trying to link these with mammalian defenses.

Defenses from bacteria are probably not conserved to humans From the point of view of bacteria, typical pathogens are viruses (phages) that are able to multiply and constantly kill huge amounts of bacterial biomass. Several restriction mechanisms have been described that can recognize or inhibit virus replication. For instance, the Crispr-Cas9 system [8] allows the bacteria to remember pieces of the genomes of a virus and later annihilate viruses of the same kind by binding to their DNA. Interestingly, this mechanism is shared with some Archæa. It illustrates that bacteria have their own 'immune' system. Although these mechanisms could typically be shared via horizontal gene transfers between unicellular organisms, it is unlikely that such mechanisms would be evolutionarily conserved to humans.

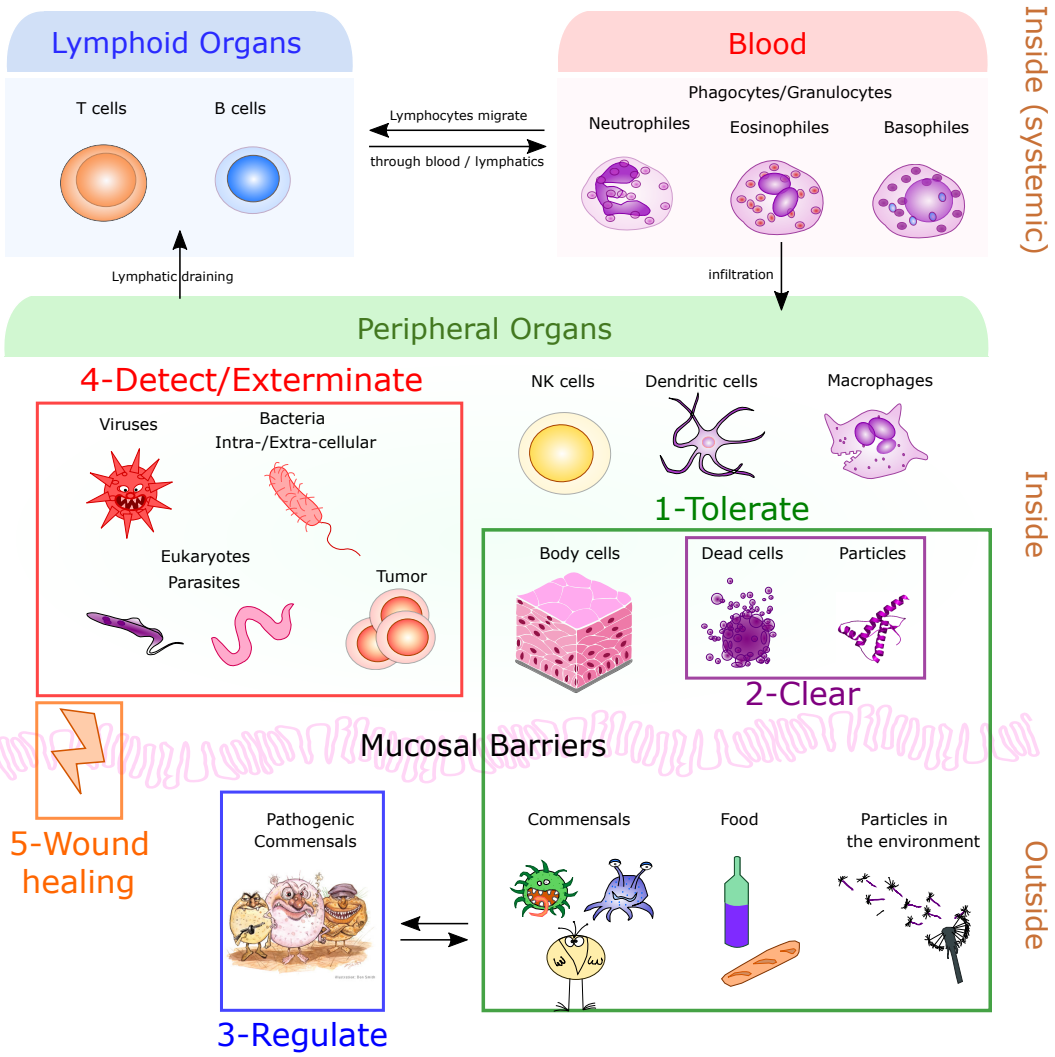

Figure 1.1: **Functions of the immune system and location of the main immune cells types. 1/ Troublemakers and associated tasks:** The immune system faces a wide set of entities, starting from healthy cells and tissues that are obviously tolerated to avoid autoimmunity. Next come dead cells and extra-cellular particles such as amyloid filaments (which cause Alzheimer's disease) that occur naturally but are constantly cleared by immune cells such as macrophages (or microglia in the brain). Although foreign particles or organisms inside organs are usually detected and cleared, thereby maintaining a sterile micro-environment as much as possible, living organisms outside the physical barriers (gut, skin, lungs, ...) can be beneficial (in the case of the microbiota) or pathogenic, depending on their type and amount. Therefore, in different environments, the same pathogen can be tolerated or attacked. The immune system has been shown to regulate the diversity of the commensals in the gut, for instance. Furthermore, most exogenous particles in the environment and food are usually tolerated otherwise generating allergies. Finally, the immune system contributes to wound healing, for instance through aggregation of suicidal neutrophiles. **2/ Cells :** Some immune cells are resident inside organs, mainly macrophages, NK cells and dendritic cells. These can detect the presence of microbial patterns (pathogen-associated molecular patterns or PAMPs) or a breakdown of homeostasis, and provide an immediate response. Mature white blood cells (T and B lymphocytes, neutrophiles, eosinophiles and basophiles) are found mainly in the blood and lymphoid organs (spleen, lymph nodes, tonsils, Peyer's patches and bone marrow), and constantly recirculate among them. In a healthy state, these cells seldom enter peripheral (non-immune) organs and inflammation is associated with infiltration of these cells inside the inflamed tissues.

Several human defenses are conserved in ancient eukaryotic organisms From the point of view of a small eukaryotic organism, like the unicellular protists, the list of troublemakers expands to viruses and bacteria. The immune defenses of unicellular eukaryotes have been very poorly investigated in literature and it is therefore hard to know whether ancestral restriction mechanisms against viruses would be conserved between protists and humans, for instance. Several clues would argue for the existence of such mechanisms. Many pathogens infecting amoebas (a kind of protist), also infect humans (e.g. Legionella, Chlamydia, etc.), suggesting they could have infected common ancestors. It has further been suggested that these pathogens 'train' inside amoeba to be resistant to antimicrobial effectors that are similar in macrophages [9]. On the other side, looking further along the path of evolution, human viral restriction mechanisms such as the family of sirtuins [10] are conserved in unicellular eukaryotes [11], though this might be caused by other functions of sirtuins. Similarly, OAS, a gene expressed downstream from interferon signaling that encodes a RNAse which degrades viral RNA, has conserved counterparts in eukaryotes [12], which are devoid of interferons, leaving the possibility for independent original functions other than viral restriction. A review of the evolution of antiviral mechanisms can be found in [13].

Eukaryotic species sometimes compete with bacteria to survive in their environment. For example, fungi produce a huge diversity of antimicrobial products (that led to the discovery of penicillin and other antibiotics). It is interesting to note that gut epithelial cells are also able to produce antimicrobial peptides such as defensins, cathelicidins or C-type lectins [6], which are important for regulating the microbial flora. Some of these are conserved throughout all eukaryotic species, such as defensins [14] [15]. These examples illustrate that the first layer of the immune system is performed by non-immune cells and consists of under-appreciated, sometimes evolutionarily conserved, mechanisms.

Pattern recognition receptors are the recognition tool of the innate immune system in metazoans Eukaryotic species are constantly challenged by a huge diversity of pathogens that were previously unknown to an individual, and that have the capacity to mutate and evolve quickly. However, at the scale of millions of years, different individuals from the same species are challenged by the same family of pathogens again and again. A class of receptors, called pathogen recognition receptors (PRRs), has been described based on their ability to recognize common molecular structures of pathogens, probably as an evolutionary tool to encode some conserved properties of these previous infections in the genome. Some

PRRs like the Toll-like receptors, were initially identified in Drosophila melanogaster and are conserved between a wide range of metazoans including humans [16].

Only some of the described PRRs are expressed by somatic cells of the body, whereas most of them are restricted to specialized immune cells, contributing to their activation and effector functions, and tuning the production of accurate signals, depending on the type of inflammation.

The first line of cells activated by PRRs are the macrophages, which reside inside tissues and can, in turn, clear pathogens, debris or dead cells via phagocytosis or by producing antimicrobial agents. These cells can secrete pro-inflammatory cytokines such as IFN-γ and help to recruit other immune cells in certain contexts, as well as releasing anti-inflammatory cytokines and/or contributing to wound healing in other contexts [17]. Macrophage-like cells have been described in metazoans [18], as early as sponges (the earliest remaining metazoans), which also carry certain PRRs (Toll-like receptors) and have cytokine signaling related to interferons [19].

Generally, the set of specialized immune cells activated by PRRs is broader and includes leukocytes, namely macrophages, their progenitors (monocytes), dendritic cells and other phagocytes such as eosinophils or neutrophils. These are commonly referred to as innate immune cells. It is noteworthy that metazoans up to invertebrate species have an immune system constituted of only innate cells and can successfully handle infections, meaning that, early in evolution, the innate immune system was enough to maintain a species over time – at least to the next generation ...

A list of PRRs is shown in (Figure 1.2), illustrating the diversity of innate recognition recognition molecules. For instance, some PRRs can recognize lipopolysaccharids on bacteria or double-stranded RNA which is characteristic of certain viruses, etc. The recognized patterns are called Pathogen-Associated Molecular Pattern (PAMPs).

Stimulatory Pathogen Associated Molecular Pattern (PAMP)	Pattern Recognition Receptor (PRR)	Signalling Adapter Protein	Transcriptional or Cellular Pathway Activated
Toll-like receptors (TLRs)			
Bacterial cell wall components	TLR2 homo/heterodimers	MyD88	NFκB / AP1
LPS	TLR4 (plasma membrane)	MyD88	NFκB / AP1
LPS	TLR4 (endosome)	TRIF	IRF3 / NFκB / AP1
Flagellin	TLR5	MyD88	NFκB / AP1
dsRNA	TLR3	TRIF	IRF3 / NFκB / AP1
ssRNA	TLR7	MyD88	IRF7 / NFκB
Nod-like receptors (NLRs)			
iE-DAP	NOD1	RIP2	NFκB
MDP	NOD2	RIP2/CARD9	NFκB / AP1
e.g. Pore-forming toxins, nucleic acid	NLRP3	ASC	Caspase-1 activation
Retinoic acid-inducible gene I-like receptors (RLRs)			
dsRNA	RIG-I	MAVS	IRF3 / AP1 / NFκB
C-type lectin receptors (CLRs)			
β-glucans	Dectin-1	Syk	NFκB

PAMP	PRR	Adapter	Cytokines
Toll-like receptors (TLRs)			
dsRNA	TLR3	TRIF	IFN-β/ pro-inflammatory
G/U rich ssRNA	TLR7	MyD88	IFN-α/ pro-inflammatory
G/U rich ssRNA	TLR8	MyD88	IFN-α
unmethylated CpG DNA motifs	TLR9	MyD88	IFN-α
Nod-like receptors (NLRs)			
ssRNA	NOD2	MAVS	Type I IFNs
Viral RNA	NLRP3	ASC	IL-1β/IL-18
Retinoic acid-inducible gene I-like receptors (RLRs)			
5′ triphosphate ssRNA	RIG-I	MAVS/ASC/CARD9	Type-I IFNs/ IL-1β

Figure 1.2: **A list of PRRs inside or at the surface of cells, and the associated patterns (PAMPs) recognized.** The bottom section focuses on viruses. Taken directly from: Liaunardy Jopeace (Intechopen.com) Pattern Recognition Receptors and Infectious Diseases. ds, double-stranded; ss, single-stranded.

Adaptive receptors in vertebrates The detection of pathogens by PRRs has some limits. First, not many genes encode PRRs in comparison to the number of pathogens and their capacity for mutation. It means that the adaptions from the pathogen side are likely to remain undetected, allowing viruses to hide by mutating critical epitopes. Second, in the case of the gut microbiota, PRRs are unable to distinguish symbiotic bacteria from pathogenic ones as long as they carry the same molecular patterns. Finally, PRRs are unable to discriminate tumors.

In vertebrates, additional immune cells, referred to as adaptive immune cells, have been described as carrying a diverse 'anticipatory repertoire' of receptors, meaning that different cells can carry different receptors for which the sequence and specificity are not directly encoded in a gene, but instead are generated via random recombination. Two such parallel systems have been described so far: in jawed vertebrates, the T cell receptor and B cell

receptor (TCR and BCR) gene segments can rearrange themselves at the chromosomal level during T and B lymphocyte development respectively (as shown in (Figure 1.3) for BCRs), creating a huge variety of receptors: more than 10^{14} different TCRs, although not all are generated in one individual [20]. Separately, in jawless vertebrates, leucine-rich protein modules can assemble in a combinatorial manner and act as adaptive receptors [21].

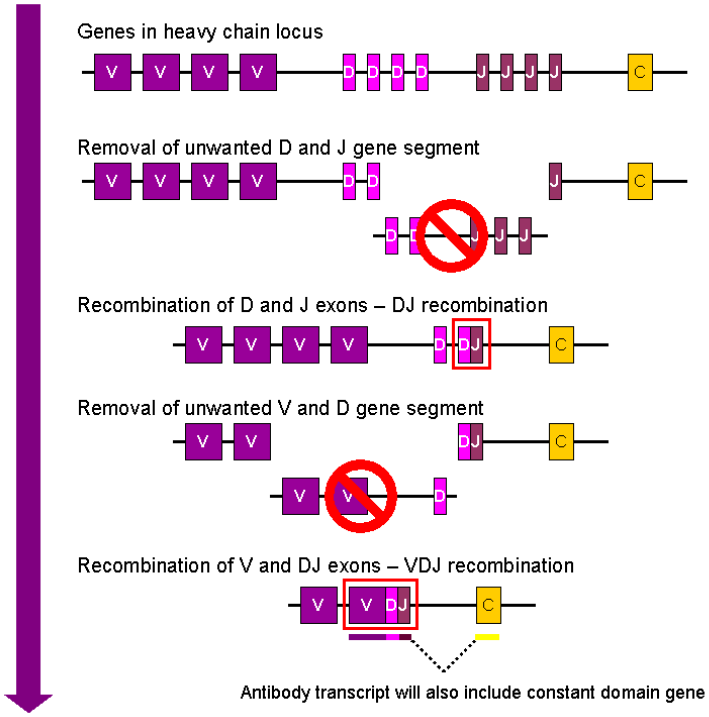

Figure 1.3: **Mechanism of random receptor generation for the B cell receptor (BCR) in jawed vertebrates.** Germline cells and B cell precursors have complex chromosomal loci for the chains of the BCR gene (namely the Igκ, Igλ and IgH genes) with repetitions of sequences called the V, D, J and C regions. For instance, the IgH locus contains 40 different V regions, 25 D regions and 6 J regions. During their development, B cells proceed to recombination, in which only one V, D and J segment each is kept for one copy of each of these three genes, while other segments are removed and lost. The junction of segments is performed by an enzyme (terminal deoxynucleotidyl transferase) that randomly adds nucleotides, enhancing the possible number of receptors that can be generated. Taken directly from Wikipedia/Antibody.

Role of lymphocytes in the immune system (of jawed vertebrates) The recognition mechanisms and effector functions of B and T cells are distinct and complementary.

The BCR of B cells can recognize 3D structures, mainly proteins or native antigens, but also sugars and lipids. When the receptor recognizes an antigen with high affinity, the cells expressing these receptors get activated, proliferate and differentiate to effector or memory cells. Finally, effector B cells (plasmablasts) have the capacity to release antibodies (copies of their BCR), which diffuse throughout the body and fix native antigens and the pathogens themselves (opsonization).

The TCR of T cells, in contrast, recognizes peptides when they are presented on a MHC complex (Figure 1.4A). After peptide-MHC complex recognition, similarly to B cells, the cells proliferate and become effector or memory cells. T cells can be separated into (1) cytotoxic T cells (CD8+), which can kill infected cells via contact-dependent mechanisms; (2) helper T cells (CD4+, with the $\alpha\beta$ type of TCR) that produce cytokines to boost CD8 T cells, B cells and innate immune cells. Additional types of T cells, and those carrying TCRs that recognize other molecules than MHC complexes, such as NKT cells, are not counted in the 'classical' T cells.

MHC complexes allow cells to present fragments of their intracellular proteic content at the surface. All cells of the organism express Type I MHC complexes, which are loaded with fragments of their proteins, even at steady state. These complexes can be recognized by CD8 T cells, which implies that every cell from the body can potentially be killed by a cytotoxic T cell.

However, only certain/specialized types of cells carry MHC II complexes to high enough levels to activate CD4 helper T cells. These cells are called antigen presenting cells (APCs). These encompass B cells and innate immune cells (notably dendritic cells) located within tissues. These cells can also present antigens from the pathogens found in the tissue to their MHC II (Figure 1.4B).

T cells are a more recent evolutionary layer than the innate and somatic immune layers, but they rely on the presence of APCs and innate signals to be activated.

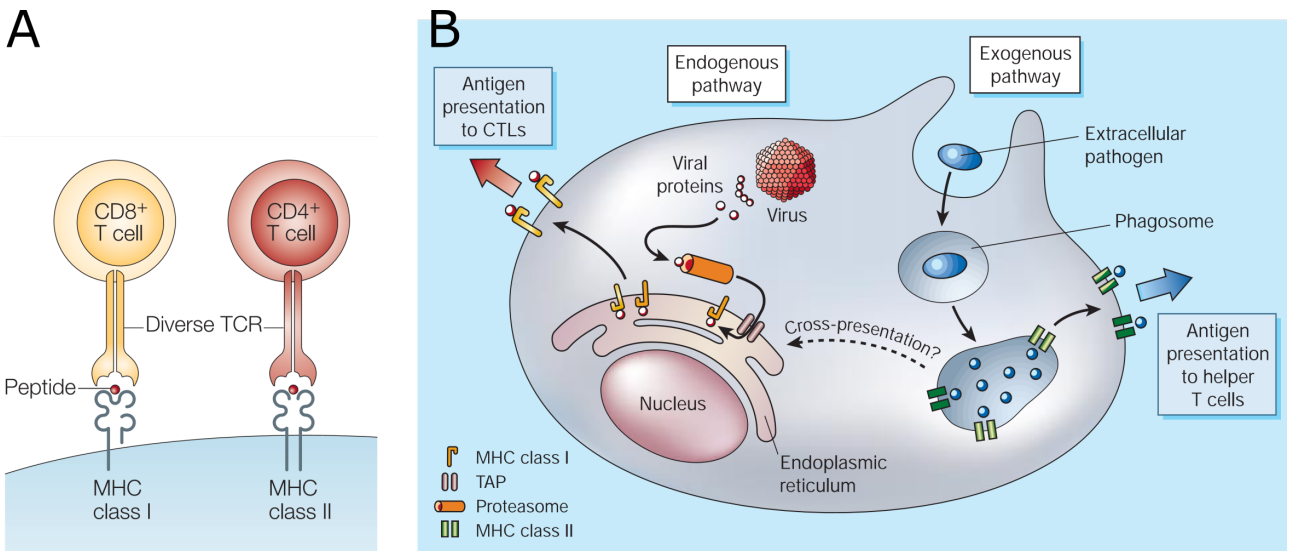

Figure 1.4: **Recognition of antigens by T cells and principles of antigen presentation.** A. Each T cell carries one type of (recombined) TCR with multiple copies at its surface. The TCRs recognize peptides when loaded onto MHC complexes. CD8+ T cells recognize peptides on Type I MHCs in contrast to CD4+ T cells, which recognize them on Type II MHC complexes. B. Antigen presentation: All cells can process intracellular proteins, healthy proteins as well as those from viruses or intracellular bacteria, onto MHC I complexes, which can activate specific CD8 T cells (CTLs), but only antigen presenting cells (APCs) are able to cross-present extracellular fragments onto their MHC II complexes, which can also activate CD4 T cells. Taken directly from A: Van Kaer 2005 (Nature Reviews Immunology) [22] and B: "Immunology: Professional secrets", Craig R. Roy 2003 (Nature) [23]

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The main advantage of carrying a repertoire of recombined receptors is the capacity to recognize antigens (potentially all possible 3D epitopes for B cells and small peptides for T cells) that the individual or even the species never saw before; and to clonally expand the cells with appropriate receptors when they are needed. Therefore, by generating B and T cells with a long-lived memory after infection, the body can keep track of the infection history of the individual by maintaining a specific set of useful TCRs and BCRs. It is contributing to the 'immunization' principle, where the immune system becomes stronger against a pathogen after a first infection.

On the other hand, recombined receptors could recognize 'antigens' from our own body, leading to autoimmunity. Several tolerance mechanisms have been described. First, the development of B and T cells is controlled by positive and negative selection mechanisms in the spleen and bone marrow [24], and in the thymus [25], respectively, a process called 'central tolerance' that ensures that cells activated by self-antigens are deleted during their development. Central tolerance is not perfect and mature auto-reactive cells can be detected in the periphery [26, 27]. Luckily, several mechanisms are controlling the physiological effect of these escaped cells:

1/ First, the development of T cells carrying an autoreactive TCR can be skewed into parallel lineages than the 'conventional' T helper cells. Cells with high self-reactivity can differentiate into thymic regulatory T cells in the thymus (tTregs, or natural Tregs nTregs) [28], which express the transcription factor Foxp3 and can suppress the immune response by many complementary mechanisms [29]. Other specialized populations of T cells, showing high self-reactivity, have been described and are discussed here [30]. Here, unless otherwise stated, we use the term helper T cells to specify conventional helper T cells, meaning cells originating from Foxp3- mature T cells (and carrying the TCR$\alpha\beta$). tTregs are outside the focus of this study.

2/ Second, a phenomenon called "peripheral tolerance" [31] have been observed in vivo, and includes the deletion of auto-reactive cells when they are activated in a non-inflammatory context; the release of anti-inflammatory signals by immune cells; and especially the suppressive mechanisms of tTregs. Conventional T cells can also contribute to peripheral tolerance by the secretion of anti-inflammatory cytokines such as IL10 and TGFβ. It is the focus of the present study to understand how a T helper cell decides to behave in a pro- or anti- inflammatory manner, and in particular, to express specific cytokines.

As another source of self-reactivity, activated B cells are characterized by the ability to specifically mutate the gene of their receptor, a process called somatic hypermutation [32,

33]. For the common type of B cells (follicular B2 cells), this process is spatially contained in structures called germinal centers, where the selection process ensures that the affinity of the BCR to the antigen increases with time while not recognizing self-antigens. Helper T cells also have a crucial role in this process by producing signals that support B cell proliferation and survival, and by selecting the fittest B cells for the antigen [34]. These helper T cells are called T follicular helpers and are also left out of this work because of the lack of commonly acknowledged protocols for generating them in vitro, yet.

The effector functions of B and T cells are illustrated in (Figure 1.5). It is important to note that B cells can access native antigens in the extracellular milieu via the BCR but cannot detect viruses or bacteria when these are intracellular. The effector fucntions of B cells are, consistently, extracellular (through the production of antibodies). CD8 T cells, on the other hand, can scan the intracellular content of cells via the peptides presented on their MHC I, but cannot bind native antigens. When CD8 T cells kill infected cells, they contribute to kill the intracellular pathogens inside. CD4 T cells are more complex, as they can access peptides derived from both intra- and extracellular pathogens via antigen presentation and cross-presentation, and as they boost the response of both CD8 T cells (intracellular) and B cells (extracellular), among other functions like recruiting other cell types.

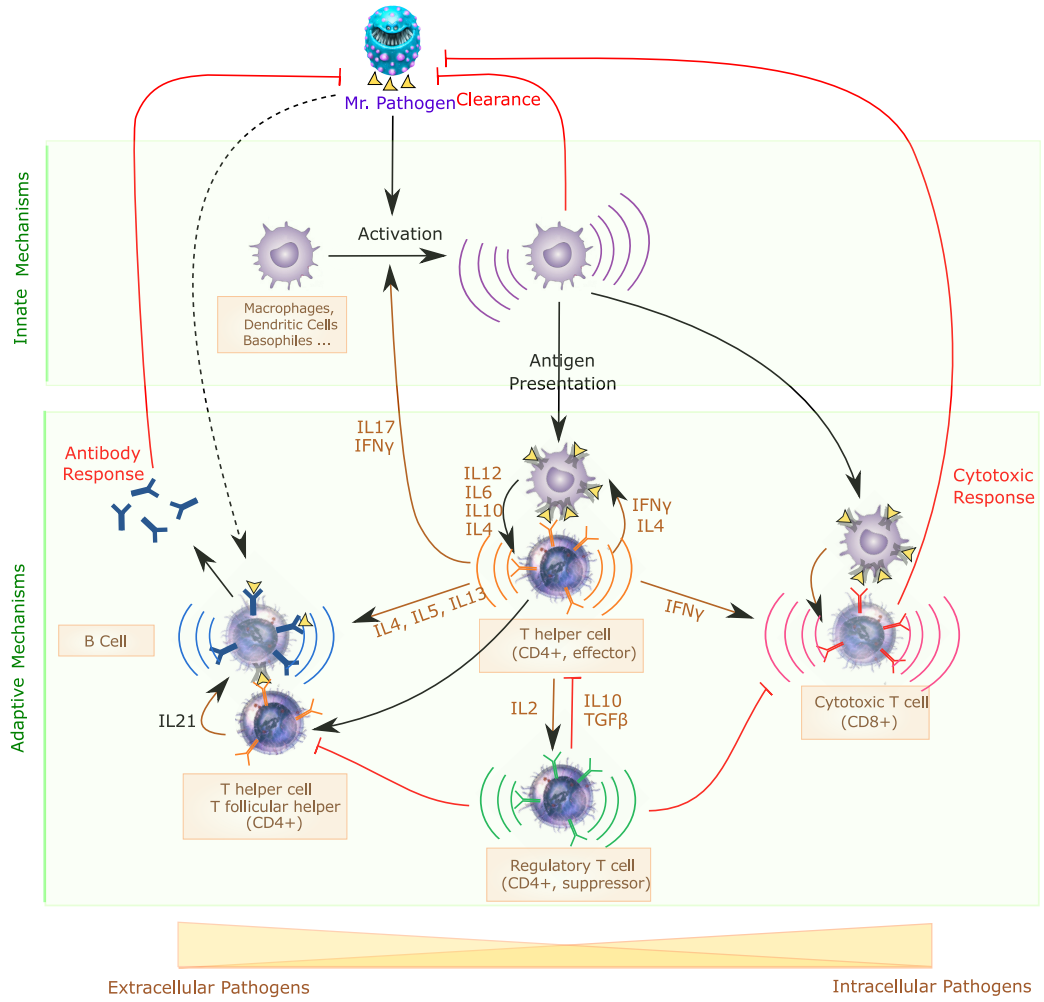


Figure 1.5: **Central role of helper T cells in the adaptive immune response.** The activation of T cells relies on the interaction with antigen presenting cells (APCs). They recognize pathogens with their PRRs in the periphery, become activated, migrate to lymphoid organs, and cross-present peptide fragments of the pathogen to CD4 and CD8 T cells. B cells, in contrast, can directly bind native antigens that are brought to the lymphoid organs, or that are bound to previously present antibodies. Activated T and B cells follow a clonal expansion phase before migrating via the blood to the infected organ. As effector functions, CD8 T cells can directly bind and kill infected cells that display peptides from the pathogen on their MHC I. B cells release antibodies that diffuse throughout the whole organism (even outside the gut mucosa) and directly bind epitopes of the pathogen, helping to neutralize it and to recruit innate cells to clear the area. Finally, CD4 T cells or 'helper T cells' play a central role by producing a set of cytokines that are necessary to boost different immune compartments. By production of IL-4, IL-5 or IL-13, they enhance the B cells' response; by producing IFN-γ, they boost the CD8 response and antigen presentation and support macrophages, and innate cells in general; by producing IL-17, they help to recruit massive amounts of innate cells such as neutrophiles, to the inflamed organ. Some helper T cells are also characterized by suppressive capacities and are called regulatory T cells. They can, among other mechanisms, dampen the local immune response by producing anti-inflammatory cytokines such as IL-10. A full B cell response (from convenional, follicular B cells) requires a physical interaction between B cells and specific CD4 T cells, called T follicular helpers cells.

Finding mistakes: the discontinuity theory of NK cell activation An additional immune protection layer is given by the NK cells. These are innate cells that can be activated by PRRs, and produce cytokines that mirror those of helper T cells at early time points during infection. Additionally, each NK cell carries a combination of inhibitory and activatory receptors encoded by a large set of genes, which recognize the expression of different variants of MHCs (which are highly polymorphic), as well as viral proteins mimicking MHCs [35]. Tumorous cells and infected cells have an abnormal expression of MHCs variants, caused by viral mechanisms in the latter case, called 'missing self'. NK cells constantly scan organs for the expression of specific MHC variants at the surface of cells, triggering a combination of inhibitory and activatory receptors at their surface. As long as the NK cells are inhibited by the binding of inhibitory receptors to MHCs, they are silent. However, MHC downregulation within cells removes this inhibition and triggers the activation of NK cells, allowing them to detect tumors and infected cells. Interestingly, the NK receptors are sensitive to the peptides presented on the MHCs, which has been suggested to additionally detect changes in antigen presentation on tumor or infected cells [36]. Based on the signaling mechanisms of the NK receptors, a theory of 'discontinuity' has been proposed, where these receptors tune themselves to an average amount of MHC expression so they can react to an abrupt change rather than the absolute expression level of MHCs [37].

A layered view of the immune system As a summary of this part, the main cellular actors of the immune system are represented in layers (Figure 1.6), by separating somatic cells, innate and finally adaptive immune cells.

The distinction between innate and adaptive cells historically originated from empirical properties such as innate cells being able to reply immediately to a pathogen and the capacity of adaptive cells to initiate a memory response. The innate–adaptive distinction based on these criteria has proven to be difficult in light for recent findings. For instance, NK cells are able to initiate a memory response [38] although they are considered to be innate cells; B1 cells, a subset of B cells, are able to initiate an immediate response while carrying recombined BCRs. Another way to base this distinction between innate and adaptive would be to say that innate cells carry and are activated by predefined receptors for pathogens (the PRRs) that are encoded in the germline, whereas adaptive cells also carry receptors that are somatically determined by gene recombination and for which the repertoire is different between two individuals. As every rule comes with exceptions, several T and B cell populations carry predefined subsets of rearranged receptors that can recognize predefined

structures that are not necessarily peptide–MHC complexes [39], making it hard to draw a precise line between innate and adaptive cells. Such cells (NK T cells, or cells recognizing specific, non-MHC structures) are likely to be conserved vestiges of evolutionary steps between initial multigenic receptor gene families and later recombined receptors.

Even though most of the cytokines produced by helper T cells can also be produced by other cell types (for instance by NK cells or dendritic cells), helper T cells have become indispensable for both boosting and regulating the immune response. For instance, the depletion of CD4 T cells due to HIV infection, or T cell deficiencies lead to immuno-suppression, whereas a lack or deficiency in regulatory T cells leads to lethal autoimmunity. Therefore, during an immune response, T cells have a central role and the progeny of only a few helper T cells with the appropriate TCR are required to ultimately produce the right cytokine at the right place and at the right time.

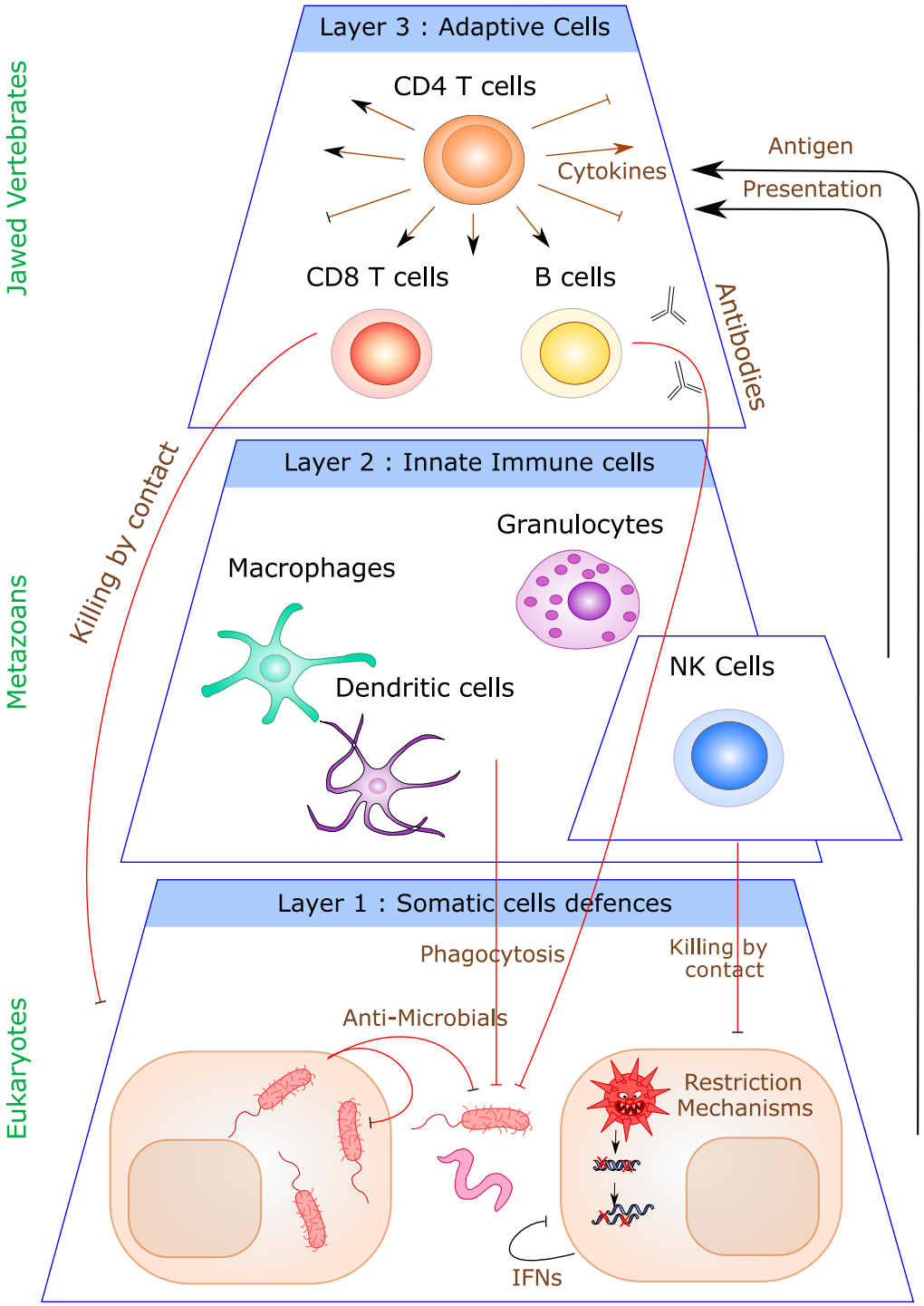


Figure 1.6: **An evolutionary pyramid view of the immune system.** The first (bottom) layer is the somatic cells of the body own defenses, some of which are conserved in other eukaryotic species. For instance, body cells can produce interferons or antimicrobial agents, or contain viral infection by restriction mechanisms such as RNA interference or by helicases that cut double-stranded RNA, proper to viruses. Next, innate cells can scan the organs, recognize pathogens through PRRs and clear pathogens, as well as launching inflammation by producing cytokines. Such cells can be seen in all metazoans. Finally, in jawed vertebrates, adaptive immune cells, activated by innate cells, help to clear the pathogens. In this pyramid, helper T cells can be put at the top because they regulate all other components by the production of cytokines, while being activated by lower stages.

1.3 Differentiation of helper T cell subsets

In this work, we are investigating how T helper cells decide to differentiate and produce a particular set of cytokines. The properties of T helper cell differentiation, regarding the signals they receive and the cytokines they produce are developed in this part. The mechanisms underlying the fate decision will be presented in the next section, and are the basis of the mathematical model presented in Chapter 3.

Helper T cells arise from naive cells Freshly differentiated mature T cells leaving the thymus are characterized by surface markers that are lost upon activation and are called 'naive T cells'. They recirculate between the blood and lymphoid organs, with a long lifespan (up to years), until they finally recognize a peptide–MHC complex with high affinity. Once they meet their cognate antigen, they get activated, they can acquire the markers of effector cells, producing high amounts of cytokines, or long-lived memory cells, which can be re-activated much faster than naive cells upon re-infection.

Subsets of helper T cells and their impact Depending on the type of pathogen and the micro-environment, naive CD4 T cells can differentiate into different subsets of helper T cells with specific patterns of cytokine expression: Th1 can be defined by the production of IFN-γ, necessary to support the CD8 response and immune responses against intracellular pathogens [40]; Th2 can be defined by IL-4 production, and support B cell responses and extracellular pathogen clearance [41]; Th17 cells which notably produce IL-17, and which, in turn, promotes the recruitment of innate cells and the clearance of some fungi and extracellular bacteria [42, 43]; Tfh, defined by their presence inside germinal centers together with the expression of CCR5 and the production of IL-21, and that are necessary for the maintenance of germinal centers via direct costimulation of B cells in a CD40-CD40L dependent manner [44]; Th9 cells, which produce IL-9 but no or low IL-4 [45, 46], and whose role is not fully delineated yet because Th9 cells seem to have both pro- and anti-inflammatory properties, and have been linked with several pathologies [47].

Other cytokines can be produced by helper T cell subsets depending on the inflammatory context, the pathogen and the type of APC encountered. For instance, Th2 often produces IL-5 and IL-13, and Th17 sometimes produces IL-22, but these cytokines are not included in their definition, as they are not always co-expressed with the major cytokine. Additional names have been given to cells based on the production of such cytokines: Tr1 cells which produce IL-10 but do not express Foxp3; Th22 cells, which produce IL-22 but not IL-17; and

Th3, which produce TGF-β but do not express Foxp3 or IL-10.

Finally, naive T cells can differentiate into Foxp3+ Tregs. These cells are called iTregs when differentiated in vitro, and 'peripherally derived Foxp3+ Tregs' (pTregs) when they arise in vivo. They are defined by the expression of Foxp3, and can harbor suppressive properties [48] (Figure 1.7). The extent to which they mirror the population of 'professional' Tregs derived from the thymus (nTreg or tTregs) is still not fully appreciated, and pTregs and tTregs do not suppress in the same situations. The definitions of regulatory T cell can be multiple depending on the studies. In a functional basis, a T cell that has suppressive capacities in vitro or in vivo can be called a Treg, and this definition would include Tr1 and Th3 cells depending on the experimental setting. In this work, the iTregs or tTregs implicitely refer to 'Foxp3+ iTregs' or 'Foxp3+ tTregs', respectively, and 'Tregs' englobes iTregs and tTregs.

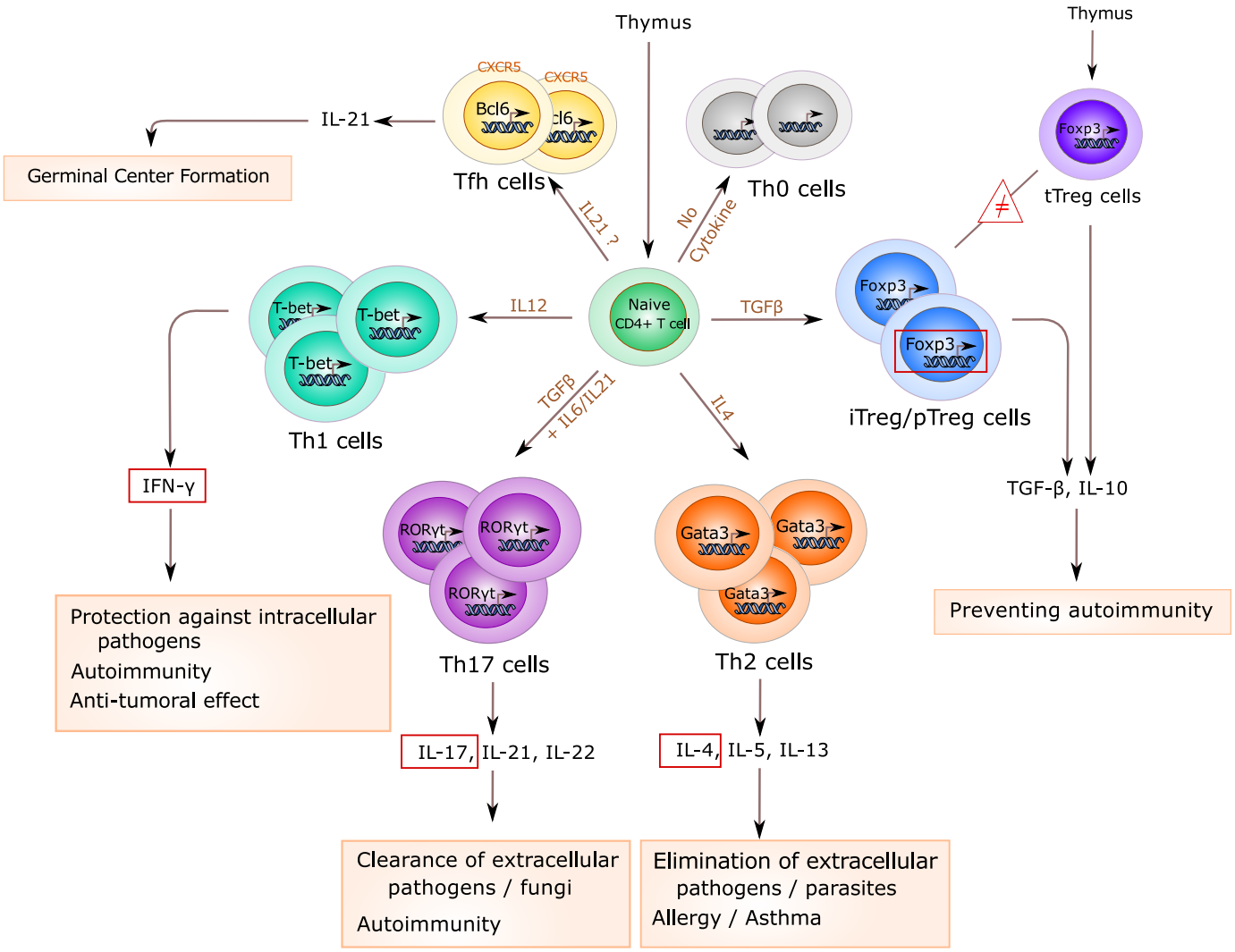


Figure 1.7: **Major helper T cell subsets.**The major subsets arising from naive T cells are depicted, along with the property defining them (red box), the cytokines they commonly co-produce and their main functional pathogenic targets. The 'canonical' cytokines that can induce the differentiation of naive CD4 T cells into to each subset in vitro (except for Tfh) are shown in brown. Note: in vivo, differentiation can happen in response to other cytokines or combinations of inducing signals, depending on the inflammatory context.

Therapeutical impact of helper T cells Autoimmune diseases such as multiple sclerosis or rheumatoid arthritis are linked with an excessive Th17 response [49] and Th1 cytokine profiles [50], consistent with murine models of susceptibility to autoimmunity [51]. Th2 cytokines can lead to asthma and cause atopic disease [52]. Tregs inside tumors contain detrimental suppressive effects [53] and Th1 cells can restrict tumor progression [54]. In contrast, Th17 cells can promote or inhibit a tumor, depending on the context [55, 56], but it has to be kept in mind that IL-17 can also be produced by other cells such as $\gamma\delta$ T-cells in these contexts.

Manipulating the T cell responses is a tantalizing target for anti-tumor treatment or for the re-establishment of a balanced immune system. Therapies can involve cytokine injections or blocking antibodies against specific cytokines, but these have to be cautiously administered because of unpredictable pleiotropic effects [57]. As an example, systemic administration of anti-IL-17 had strong side-effects on the inflammation status of the gut [58] and direct sub-cutaneous skin administration of anti-IL-17 against psoriasis is preferable [59]. Another emerging strategy consists of engineering T cells from a patient before grafting them back, avoiding the risk of graft rejection. For instance, anti-tumor "adoptive immunotherapies" consisting of redirecting T cells to recognize a tumoral antigen through the expression of a chimeric antigens receptors (CARs)) which has shown promising effects for tumor rejection in melanoma and B cell malignancies [60, 61].

Although helper T cell subsets are commonly accepted as separate lineages, because they keep a memory of their cytokine profile [62] upon TCR restimulation without the addition of specific cytokines in vitro, they show a high degree of plasticity in vivo [63]. A transferred anti-tumor Th1 cell can become a Treg and show unwanted or opposing effects. Similarly, targeting autoimmune diseases by transferring Tregs in mice is challenged by the poor stability of these iTregs in vivo [64, 65] .

Therefore, understanding the mechanisms controlling helper T cell differentiation and plasticity, and how to induce stable helper T cells subsets in vitro can help us to design or improve immunotherapies.

Signals impacting the differentiation of naive T cells into cytokine producers The activation of naive T cells happens via interaction with one or more APCs over time, providing the T cell with several signals at the same time, namely (1) TCR stimulus [66], (2) different contact-dependent costimulatory signals [67] and (3) the cytokines secreted by the APCs or that are present in the medium [68] (Figure 1.8).

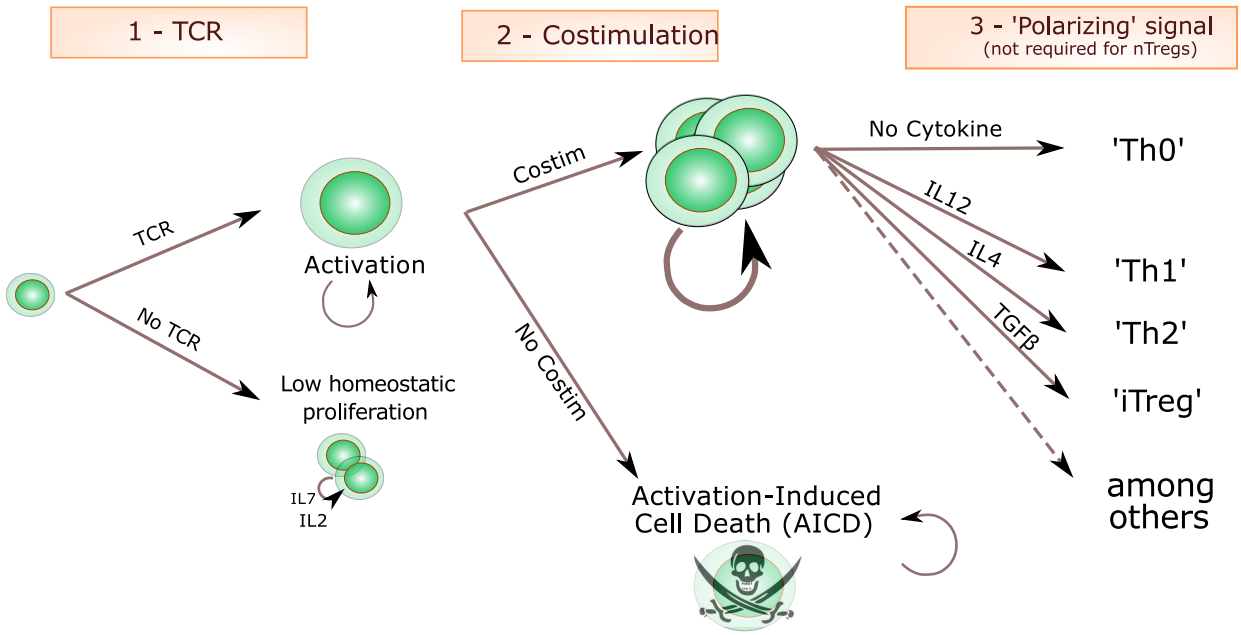


Figure 1.8: **The 'three signals theory' of the simultaneously received signals required for the activation of naive T cell by APCs.**The activation of the TCR of naive T cells is enough to activate them whereas they undergo homeostatic proliferation in the body without encountering high affinity ligands. Upon activation, costimulatory signals are needed to support the full activation, proliferation and productions of cytokines, whereas TCR activation without costimulation can lead to death, called 'activation-induced cell death' (AICD) in certain conditions. Costimulatory signals are diverse and show a high synergy with TCR signaling pathways, meaning that low TCR activation can be compensated by high costimulation and vice versa. Finally, the cytokines encountered during activation have a high impact on the differentiation program and the cytokines produced later by an activated T cell. Other signals impact differentiation, such as the metabolic micro-environment, the presence of soluble molecules and appropriate localization following chemotactic signals; this list could probably be expanded.

The inducing signals responsible for in vivo differentiation into each subset have still not fully been characterized. Based on experiments using knock-out mice, several cytokines have been shown to be necessary for the initiation of a helper T cell subset and the clearance of a pathogen, but one has to remember that these experiments are pathogen-specific. For instance, in vivo, for most intracellular pathogens, IL-12 is necessary to induce an appropriate Th1 response and clear the infection [69, 70]. However, in specific cases, IL-12-deficient mice could clear viruses or bacteria and develop Th1 cells [71, 72]. In a human setting, this process can be Type I interferon-dependent [73], meaning that Th1 differentiation is not always mounted via the same signals. IL-4 deficiency leads to the incapacity to initiate a Th2 response [74]. IL-6-deficient mice had impaired Th17 response [75]. Finally, mice with targeted disruption of TGF-β signaling in T cells developed lethal autoimmunity. Although these mice had high amounts of tTregs, the peripheral maintenance (and probably conversion) of naive cells into iTregs was inhibited [76].

Consistently, activation of naive T cells in vitro in the presence of IL-12; IL-4; a cocktail of TGF-β and IL-6; and TGF-β, successfully manage to generate Th1, Th2, Th17 and iTregs, respectively. These signals are referred to here as 'canonical' inducing signals, and will be used in this study.

However, the cytokinic micro-environment in vivo is likely to be more complex, rather than restricted to only one cytokine, and probably contains opposing signals at the same time. It is possible that different infections could lead to yet unappreciated combinations of cytokines, leading to the same helper T cell subtype but with additional specific properties. For instance, Th1 or Th17 cells can express IL-10, depending on the context, which endorse them with regulatory properties [77, 78]. Studies comparing helper T cells generated by different pathogens or using reporter tools to follow the cytokines received by T cells in vivo would give insight into this new layer of complexity.

Origin of inducing signals: translating the infection type into a helper T cell subtype

Helper T cells can be seen as a standardized immune component that is activated in any type of infection, integrating a large range of signals and then controlling the other immune layers. However, one has to remember that the signals inducing differentiation are produced by APCs, meaning that helper T cell differentiation is actually manipulated and controlled by the APCs and the micro-environment where they interact. A large range of APC subsets has been described and is currently expanding, based on their localization, the cytokines they produce and their surface markers, meaning that the APC layer strongly depends on

the pathogen [79] and the location of infection (and is not standard to all pathogens like the T cell layer was). This way, at the initiation of an infection, a specific kind of APC is activated, migrates to the lymphoid organs and can provide clues to the T cells about the properties of the pathogen and the site of activation. For instance, although both pathogens lead to a Th1 response, viruses and intracellular bacteria lead to the production of different signals by APCs. It has also been suggested that T cells are instructed to migrate to different organs depending on the APC subset [80]. Further, several types of APCs have tolerogenic properties and modulate the strength or type of helper T cell subset generated [81, 82]. This is a new complex and expanding field, though a complex one, because each pathogen infection requires the study of different APCs and goes beyond the complexity of the standardized helper T cell response.

1.4 Plasticity of helper T cells

A striking property of T helper cells is their capacity to re-differentiate between subsets in specific contexts. In this work, experiments were performed to assess the early plasticity of T helper cells in vitro, and were used to test the predictive power of our mathematical model. An introduction to T helper plasticity is given here.

While T helper subsets are defined as lineages because they keep the memory of their cytokine profiles upon TCR restimulation, accumulation of evidence show that they are plastic and can reprogram into another subset in vivo. Plasticity can arise in two manners: 1/ full redifferentiation from one subset to another one, based on the cytokine expression profile and 2/ acquisition of additional cytokines or transcription factors, leading to 'mixed states' populations, endorsed with the properties of two subsets.

Here, as a non-thorough illustration, several cases of T helper plasticity have been described for each subset, illustrating the limits of the "independent lineages of T helper cells" paradigm:

Cases of stability for Th1 and Th2: In vitro generated (TCR transgenic) Th1 and Th2 cells, transferred into a WT mice, maintained their cytokine profile at different time-points after transfer [83], though at later points (140 days), their cytokine production was reduced. It shows that, without infection nor TCR activation, cells can keep memory of their cytokine profile in vivo. Generally speaking, memory cells with a Th1, Th2 and Th17 profile have been described, and they can maintain their cytokine pattern upon restimulation.

In vitro, cell activated under Th1 or Th2 condition showed plasticity to express the cy-

tokine profile of the other subset when the inducing condition was changed at an early time points. [62]. However, this article concluded that, after 4 divisions, cells were not plastic anymore to Th1/Th2 repolarization.

In [84], in vitro, after a week under polarizing conditions, Th1 or Th2 cells could be re-differentiated into IL-4 or IFN-γ producers respectively, with some cells co-producing IL4+ IFN-γ+ cells called 'Th1/2 cells'. However, when in vitro derived Th1 or Th2 cells were re-stimulated under their polarizing conditions every week for three weeks, they were much less plastic and mainly kept their phenotype upon repolarization. Finally, long-term established clones were not plastic anymore and could not be re-differentiated.

Example of stability while acquiring of a new phenotype: 'Continuous' IL-4+ IFN-γ+ cells emerge in vivo and in vitro. [These IL-4+ IFN-γ+ T-bet+ Gata3+ cells are called here Th1/2]. In [85], following adoptive-transfer of Th2 (IL-4+ Gata3+) differentiated cells into mice, and infection with LCMV (chronic, Th1 inducing virus), Th1/2 cells, co-expressing IL-4 and IFN-γ were detected and persisted as memory cells in vivo, consistently with [86]. Altogether these data suggest that, in vivo, T helper responses naturally generate Th1/2 cells. In vitro, Th1/2 can also be obtained by combination of IL-4 and IL-12 [87] while the canonical differentiation protocols use blocking antibodies and are optimized to raise only a 'clean' Th1 only or Th2-only phenotype.

Reprogramming of Th1 to other subsets can happen in vivo In [88], murine Th1 cells were polarized in vitro and adoptively- transferred into Rag-/- mice. Some adoptively-transferred Th1 cells were converted into IL-17+ IFN-γ- cells, showing that Th1 to Th17 trans-differentiation can happen in vivo in a lymphopenic mice.

Plasticity from the Th1 phenotype to the Treg phenotype has been described, after the co-transfer of human Th1 cells with human T cells transduced with PDL1, a marker expressed by tumor cells but not only, into NSG humanized mice [89].

In [90], murine in vitro generated T helper cells were adoptively transferred, into a WT host, prior to the challenge with an helminth infection (Th2 response prone pathogen). Adoptively transferred Th1 or Th17 cells were redirected to produce IL-4, while only a few cells kept producing IFN-γ and IL-17, respectively, showing a complete trans-differentiation for most of the cells. Interestingly, the Th1 to Th2 conversion in vivo also occurred in IL-4/IL-13 double deficient mice, though in this case, most of the cells kept IFN-γ production and became Th1/2 cells. Strikingly, transferred iTregs did not express IL-4 following helminth infection.

Th17 cells can win the 'plasticity awards' In vivo, using a fate-mapping approach to track IL-17 expression, [91] showed that a high percent of cells that previously expressed IL-17 had lost its expression in steady-state (called 'exTh17' cells). After inducing inflammation by injection of anti-CD3, these exTh17 cells could become IL-10 producers with regulatory capacity, similarly to Tr1 cells.

In vitro, the restimulation of human Th17 cells in the presence of IL-12 has been shown to induce the generation of IL-17+ IFN-γ+ 'Th1/17 cells'. These Th1/17 cells co-express Rorγt and T-bet and were also found naturally in human blood [92]. Interestingly, murine ex vivo Th17 cells were less responsive to IL-12 (as compared to in vitro Th17 cells) due to a lower IL-12Rβ2 expression, generating a lower percentage of Th1/17. Pre-treatment with IFN-γ upregulated IL-12Rβ2 expression and allowed a higher conversion to Th1/17 cells.

In [93], in vitro generated OTII TCR-transgenic Th17 cells were restimulated in presence of different combinations of cytokines. In the presence of TGF-β at each round of restimulation, IL-17+ IFN-γ- producing cells were stably maintained. However, when Th17 cells were restimulated with IL-12-/- APCs in presence of IL-23, a significant percentage of IL-17- IFN-γ+ cells arose. Finally, restimulation of Th17 cells in the presence of IL-12 led to the generation of 60% of IL-17- IFN-γ producers, though this did not happen in presence of TGF-β, and only a few cells still expressed IL-17. Therefore, Th17 are sensitive to IL-12 signaling and plastic to IFN-γ expression, while TGF-β acts as a stabilizer of the Th17 phenotype.

Interestingly, Th1 cells derived from Th17 [94] (called 'non-classic Th1'), keep memory of their previous differentiation status as Th17-specific gene locI such as Rorγt and CCR6, and remain demethylated [95].

In [96], Th17-derived Th1 cells were able to induce colitis in a T cell based -colitis model, while IFN-γ-/- Th17 cells did not, suggesting that IFN-γ+ Th17 cells have an inflammatory and pathogenic potential, while IFN-γ-/- (IL-10+) Th17 cells have a less pathogenic and more regulatory phenotype.

T helper cells producing both IL-4 and IL-17 have been described in human in the memory compartment of patients with atopic asthma [97, 98] and the restimulation of human Th2 memory cells with Th17 inducing cytokines allowed to generate these cells in vitro [97]. Interestingly, stimulation of human naive T cells with a combination of Th2 and Th17 inducing cytokines failed to induce both IL-4 and IL-17 but the restimulation of memory Th17 cells with Th2 polarizing cytokines did succeed [98].

Finally, isolated human Th17 TILs (Tumor Infiltrating Lymphocytes) acquired Foxp3 ex-

pression and IFN-γ production following restimulation using allogeneic PBMCs,[99]. It has to be noted that Foxp3 expression is transiently induce following TCR stimulation in humans, and is not necessarily associated with a Treg phenotype.

iTregs are not stable, and Foxp3+ cells expressing other master transcription factors can be observed in vivo, likely arising from tTregs. iTreg are known to lose Foxp3 expression in vitro over time, and, even after several rounds of polarization, they lose Foxp3 expression following adoptive transfer in vivo [65]. It has been described that Foxp3+ cells can harbor additional properties:

1. Foxp3+ T-bet+ IFN-γ+ cells can be detected in vivo, are suppressive, and relied on T-bet expression for localisation at the site of infection [100]. IFN-γ has also been proposed as a tool for Tregs to mediate suppression [101].

2. Similarly, Foxp3+ Gata3+ Tregs are suppressive in vivo, and Gata3 was required for a localization at the site of inflammation and the maintenance of Foxp3 expression. [102].

3. Foxp3+ Rorγt+ Tregs were described in the gut, are suppressive, and carry epigenetic marks specific of tTregs (demethylation at the Foxp3 locus), suggesting that they derived from tTregs. [103].

Thus, Foxp3 expression can in that way be seen as a parallel fate that mirrors all the T helper effector subsets [104] (Figure1.9).

To summarize, these findings highlight the diversity of experimental protocols for assessing plasticity, and the complexity of T helper subsets reaction when they are restimulated in the presence of cytokines (in vitro) or in an inflammatory context (in vivo). The strongest cases of stability have been shown in case of restimulation without adverse cytokines, or repeated re-polarizations. However, in the general case, differentiated T helper cells seem to be plastic. A mathematical model would help to find out whether the already known mechanisms are able to explain this complexity, or whether new mechanisms remain to be discovered.

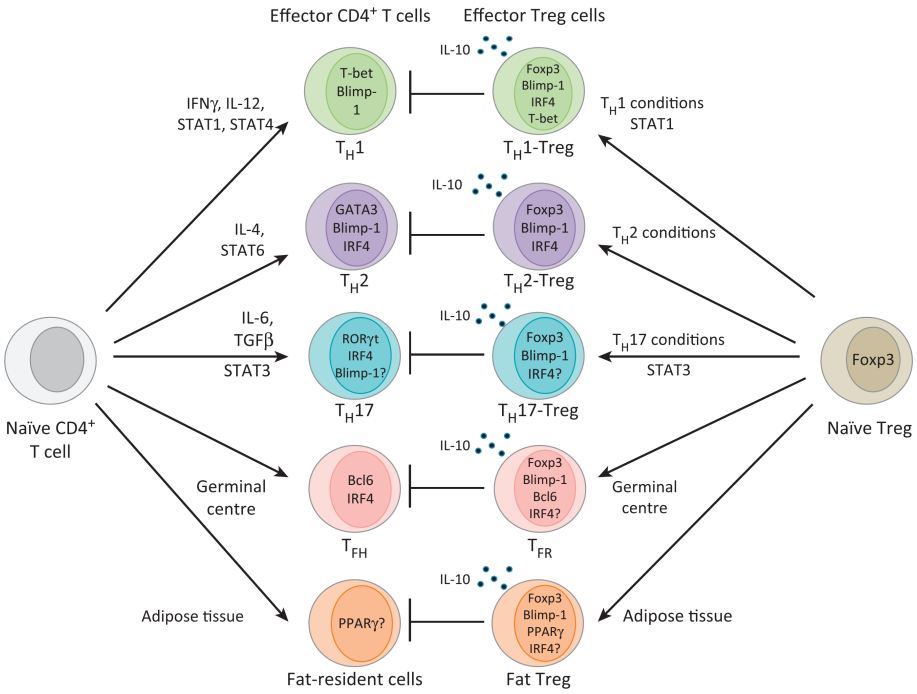


Figure 1.9: **Foxp3 expression can be seen as a parallel fate decision,** expressed together with the specific properties of pro-inflammatory T helper subsets, while maintaining suppressive activity. (Taken from [104]).

1.5 Molecular mechanisms controlling CD4 T cell differentiation

We aim to develop a mathematical model describing the kinetics of T helper differentiation. It requires to pull together a list of major mechanisms that decide for the differentiation. This part summarizes the major intracellular pathways that link the input cytokinic signals with the differentiation programs. The mathematical model was build from these published mechanisms, with simplifications, and is described in Chapter 3.

Differentiation of Th1 cells The course of IL-12 induced Th1 differentiation follows two main phases: IL-12 induction of Th1 genes and very restricted/limited IFN-γ production, followed by a phase of IFN-γ auto-stabilisation of the Th1 phenotype with massive IFN-γ production [105](Figure 1.10). It has to be noted that IFN-γ is also provided by other cell types in vivo [106].

The receptor chains for IL-12 are regulated during differentiation. ILR12β1 is constitutively expressed in substantial amounts whereas IL-12Rβ2 is only present at very low dose in murine and human naive T cells. TCR stimulation induces a slow upregulation of IL-12Rβ2 [107], further increased by IL-12 downstream signaling through STAT4 [108], creating a slow feedback loop where IL-12 signaling increases the sensitivity to its own signaling on T

cells.

Several studies detected IFN-γ signaling as required for the stable and strong expression of IFN-γ by T cells. Naive T cells stimulated by their TCR in the presence of anti-IFN-γ show no detectable IFN-γ mRNA expression (By reanalysing data published in [109]), Th1 development is impaired in IFN-γR-/- mice upon candida albicans infection in vivo [110] and is strongly reduced in vitro [111]. Finally, addition of exogenous IFN-γ increases the production of IFN-γ by T cells although in a transient manner.

In a mechanistic point of view, IFN-γ signals through the IFN-γRI-IFN-γRII receptor complex (type 3 IFN receptor), whose chains are respectively associated with Jak1 and Jak2. Upon IFN-γ binding, Jak1 and Jak2 phosphorylation leads to the binding and tyrosine phosphorylation (Y701) of STAT1 pre-formed dimers, which re-orientate into an antiparallel dimeric structure [112], locate to the nucleus and act as a transcription factor, and lead, among other things, to the direct activation of T-bet expression in synergy with STAT4 [113].

T-bet overexpression by retroviral transfection at 36 hours after TCR stimulation via anti CD3/28 antibodies was sufficient to induce the expression of IFN-γ, even into Th2 predifferentiated cells (during 9 or 22 days) [114]. In a system where the activity of T-bet is controlled through an oestrogen receptor [115], T-bet depletion leads to a loss of IFN-γ production, and IFN-γ production depends on T-bet in a dose-dependent manner. Finally, T-bet deficient cells fail to produce high amounts of IFN-γ upon IL-12 treatment. Taken together, these findings supported T-bet as a master transcription factor for Th1 differentiation, by being necessary and sufficient for full IFN-γ production in the Th1 condition.

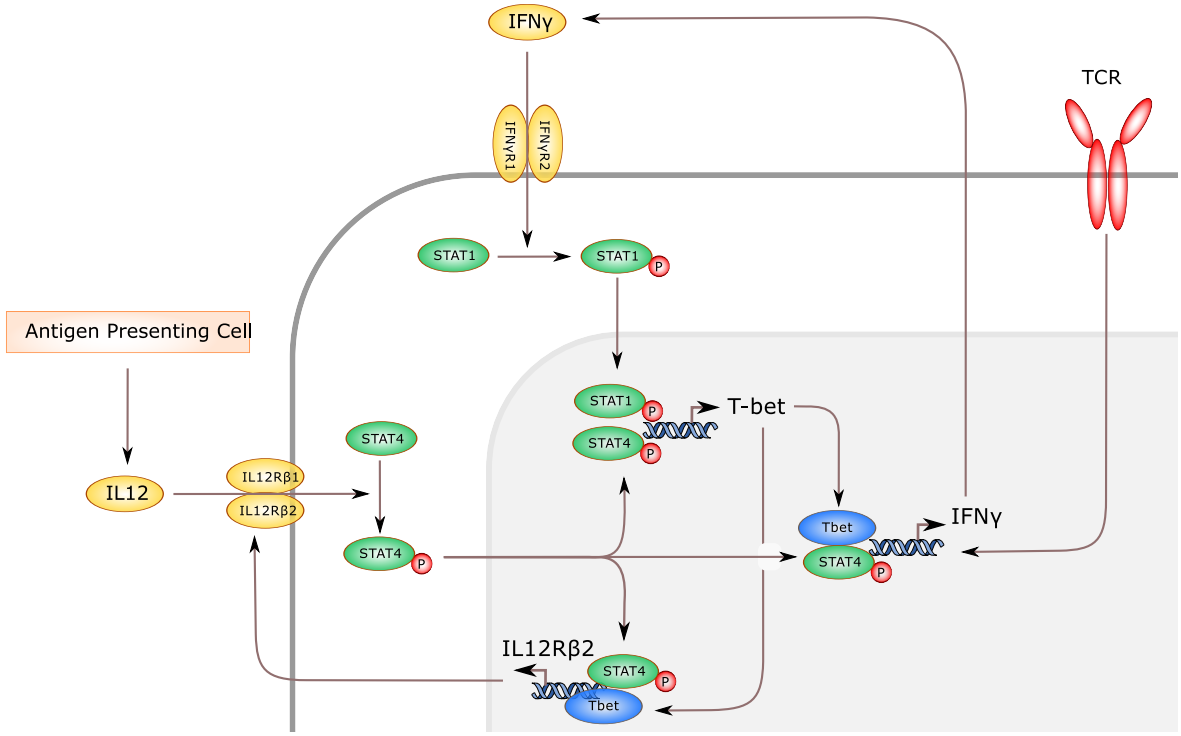


Figure 1.10: **Core Th1 differentiation network.** IL-12 induces STAT4 phosphorylation, trans-activating IFN-γ, T-bet and IL-12Rβ2, among other genes. In parallel, IFN-γ, initially produced downstream TCR, signals in part through STAT1 phosphorylation, which fully activates T-bet expression, in synergy with STAT4. This synergy can be observed by a lack of T-bet expression in the context of anti-IFN-γ, and by the fact that IFN-γ is not sufficient to trigger Th1 differentiation.

Differentiation of Th2 cells Th2 differentiation is believed to be initiated by IL-4, and further supported by the production of cytokines exerting a positive feedback by IL-4 itself and IL-13 (Figure 1.11). IL-4 as well as IL-13 deficiencies lead to an impaired response to helminths [116]. Basophiles have been suggested to produce IL-4 in vivo and to be responsible for Th2 induction [117], but the origin of IL-4 in vivo is still a matter of debate. The response against some helminths was independent of the IL-4 signaling pathway, suggesting the existence of additional Th2 induction pathways [117].

IL-4 signals by binding to the IL-4 receptor, composed of IL-4Rα and the γc common receptor, shared with other cytokine receptors. IL-13 also signals via IL-4Rα, but in complex with IL-13Rα. Another chain, IL-13Rα2 has been described to be expressed in the thymus but lacks Jaks binding intracellular regions and does not confer sensitivity to IL-13 alone [118], while it was to suggested to be part of a the IL-13 receptor complex together with IL-4Rα and IL-13Rα1 [119]. Following IL-4 binding, Jak1, bound to IL-4Rα and Jak3, to γc, are transphosphorylated, leading mainly to STAT6 phosphorylation at Tyrosine 641. In the case of IL-13, the same process happens between Tyk2 bound to IL-13Rα1 and Jak1, also leading to the same STAT6 phosphorylation [120].

Phosphorylated STAT6 proteins are able to form anti-parallel dimers and to relocate into the nucleus where they bind to STAT6 responsive elements and directly activate genes including Gata3, IL-24 and SOCS1 [121]. Interestingly, while STAT6-P also binds to the IL-4 locus and correlates with permissive epigenetic marks, it is not enough to activate IL-4, suggesting a role for chromatin remodelling rather that a direct transcriptional activation.

Gata3 is able to directly induce the expression of IL-4, IL-5 and IL-13. Gata3 deficiency in mice is embryonically lethal, but Gata3 knock-down in Th2 clones abrogated IL-4 production [122]. Finally, Gata3 overexpression in Th1 cells leads to the production of IL-4 while inhibiting IFN-γ production [123] [124], promoting Gata3 at the rank of master transcription factor for Th2 differentiation. It is of interest that Gata3 is able of activate its own transcription [125]. IL-13 and IL-4 provides an additional feedback loop to support Gata3 expression. However, IL-5 signaling in T cells has not been described, and IL-13 was shown to be insufficient to initiate Th2 differentiation, but rather to support it later, probably due to a regulation in receptor levels. TCR signaling might have an important role in Th2 differentiation by supporting early Gata3 transcription as well [126].

Additional feedback loops are evoked during Th2 differentiation, supported by Gata3 and IL-2 downstream signaling, including higher expression of IL-4Rα and IL-2Rα. IL-2 binds to the trimeric IL-2R complex, composed of IL-2Rα (CD25), IL-2Rβ (CD122) com-

plexed with Jak1, and γc, bound to Jak3. Upon IL-2 binding, Jak1 and Jak3 are trans-phosphorylated, leading mainly to STAT5 phosphorylation at Tyr 694 and switching of the STAT5 dimers into an anti-parallel structure [127], while other STATs were shown to be activated by IL-2 to a minor extent [128]. IL-2 can support Th2 development independently of IL-4 signaling [129]. However, IL-2 is not a Th2 specific cytokine because it is produced by most activated T cells in early phases, and the differentiation of Th1 cells was also shown to be dependent on IL-2 [130].

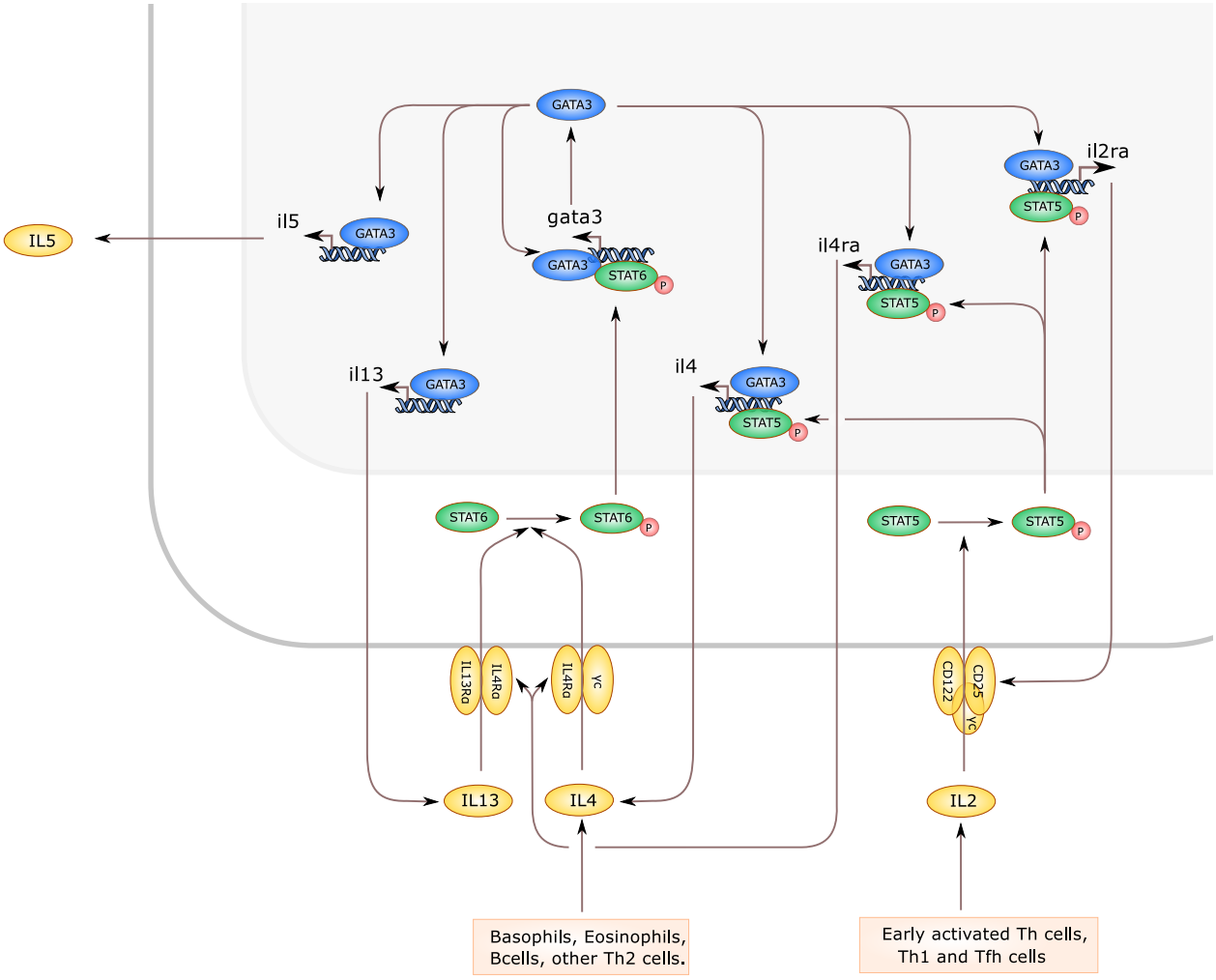


Figure 1.11: **Th2 core differentiation network.** Stat6 phosphorylation, initially triggered by IL-4, induces Gata3 expression that induces the production of the Th2 cytokines (IL-4, IL-5, IL-13), as well as IL4Rα and CD25 expression. IL-4 and IL-13 provide a feedback loop to sustain Gata3 expression, in synergy with IL-2.

Reciprocal differentiation of iTregs and Th17 cells Th17 and iTregs are both supported by TGF-β1, 2 and 3, in synergy with other cytokines. It was first discovered as a surprise because TGF-β was believed to be a pan-anti-inflammatory cytokine, and activation of T cells with tTregs and dendritic cells led to the generation of pro-inflammatory Th17 cells due to the combination of TGF-β from the Tregs and IL-6 from the dendritic cells[131]. It turned out that TGF-β can actually be pro-inflammatory in a context-dependent manner by promoting Th17 differentiation.

The main receptor for TGF-β in T cells is composed of TGF-βR1 (type I) and TGF-βR2 (type II). Several other TGF-β receptors have been actually described on T cells and might endorse the cells with other downstream signaling pathways upon TGF-β treatment: CD105 / Endoglin [132], CD109 [133, 134], and TGF-βRIII / betaglycan [135, 136]. Interestingly, there might be an unappreciated cross-talk between different members of the TGF-β superfamily ligands with TGF-β regarding T cell differentiation, for instance with Activin A [137] suggesting that they might support iTreg or Th17 in vivo independently of TGF-β. As a speculation, this diversity in the TGF-β superfamily signaling could result from the fact that TGF-β is one of the most ancient cytokines in evolution [138].

Binding to TGF-β to the classical TGF-βR1/TGF-βR2 receptor leads to the phosphorylation of Smad2, Smad3 at C-terminal serine residues by TGF-βR1. Smad2 or Smad3 can in turn heterodimerize with Smad4 and act as transcription factors in the nucleus [139], activating Foxp3. In contrast, activation of the transcription factor Rorγt downstream TGF-β, (hallmark of Th17 cells, and also activated downstream IL-6), was independent on the canonical smad2/smad3 signaling pathway [139] but was blocked by MAPK inhibitors and partially inhibited by AKT/mTOR inhibitors [140] suggesting a parallel TGF-β signaling during Th17 differentiation, potentially through CD105 [132].

Interestingly, activated T cells can produce TGF-β [141] [142], in Foxp3-dependent and -independent manners [143], though this process is more often observed on Foxp3+ Tregs. TGF-β is secreted in a latent form, initially complexed with LAP (Latency Associated Peptide), also produced from the TGF-β gene. The secretion of TGF-β requires the binding of a third protein (Latent TGF-b-Binding Protein (LTBP)), which finally allows for the release of the Latent (inactive) TGF-β into the medium. Latent TGF-β can be kept at the surface of the cells, in a membrane-bound manner via GARP (Glycoprotein A repetitions predominant) [144]. For instance, regulatory T cells express GARP upon activation which is required for their suppressive function in specific contexts [145]. Finally, the use of latent TGF-β requires enzymatic proteolysis. For instance, Tregs release active TGF-β from

its membrane-anchored latent counterpart through integrinβ8 [146] [147]. The feedback generated by TGF-β has been shown to sustain Th17 cells in vivo [148]. In general, this feedback is hard to analyze in vitro, due to the presence of high amounts of latent TGF-β in serum used for cell cultures, which can saturate the ELISA measurements of total TGF-β, and could give a source of functional TGF-β to the cells if they can activate it. A review on the relevance of TGF-β for T helper differentiation can be found in [149].

In vitro, IL-6 in combination with TGF-β is sufficient to induce IL-17 production from naive T cells, and further in vivo, IL-6 deficiency leads to a strong decrease of Th17 amounts in the lamina propria [75].

The transcription factor Rorγt (gene rorc) emerged as a master transcription factor for Th17 development. In vitro, Rorc-/- deficient cells failed to produce IL-17 in presence of IL-6 and TGF-β, while over-expression of Rorγt was enough to endorse the cells with IL-17 expression [75]. Note that Rorγ (also called Rorγ1), another isoform of Rorγt due to alternative promoter use, is mainly expressed in non-immune organs linked to metabolism, but is also induced in Th17 cells and redundantly mirrors Rorγt [150]. In vivo however, rorc (Rorγ-/- Rorγt-/-) gene deficiency did not totally abrogate Th17 differentiation, and a second transcription factor of the same family, Rorα (isoform 4), was described to act in synergy with Rorγt to promote IL-17 production [151, 152]. Rorα overexpression was enough for IL-17 expression and further co-overexpression with Rorγt lead to greater IL-17 production, even in adverse T helper conditions as Th2. The double deficiency rora-/- rorc-/- completely abrogated Th17 differentiation in vivo in a EAE model, showing that the partners Rorγ, Rorγt and Rorα4, control Th17 differentiation.

Mechanistically, IL-6 signals through IL-6Rα (CD126 / gp80) and gp130 (CD130, IL-6Rβ) homodimers receptor chains, both binding to Jak1. IL-6 induces Jak1 phosphorylation leading to phosphorylation of STAT3 [153], but also STAT1 [154], whose dimers (hetero- and homo-) act as transcription factors in the nucleus. Soluble forms of IL-6Rβ and gp130 have been observed in the human serum, and were suggested to interfere with or support IL-6 signaling (by trans-signaling) depending on the context, as discussed in [155] .

Phosphorylated STAT3 dimers transactivates many targets including IL-21, Rorc, Rora, IL-17a, IL-17f, IL-6ra, IL-23r [156] as well as TGF-β [157]. IL-21 is further secreted and signals through the IL-21R-γc receptor chains, bound to Jak1 and Jak3 respectively, and inducing STAT3 phosporylation (together with Stat1 and both Stat5a and Stat5b isoforms). Therefore, IL-21 provides a direct feedback loop to support the Th17 phenotype [158].

The balance between Foxp3 and Rorγt expression is regulated by additional mechanisms.

Among them, Rorγt inhibits Foxp3 expression by direct binding to its promoter, ensuring that Foxp3 is not maintained in the Th17 condition. Foxp3, in turn, directly binds to Rorγt, which inhibits its function [159] and its stabilization. Therefore, during Th17 differentiation, the synergy between TGF-β and IL-6 allows for sustained Rorγt expression whereas in iTreg condition, Rorγt expression is transient but not sustained.

The expression of Foxp3 induced by TGF-β is not stable. IL-2 further supports iTreg differentiation via direct binding of STAT5 to foxp3 promoter, sustaining its expression. iTreg differentiation is strongly reduced in vitro on IL-2 deficient cells [160]. It is interesting to see that iTreg cells do not produce feedbacks specific to their own subset: IL-2 and TGF-β are produced by activated T cells in general. In contrast, thymically derived tregs (tTregs or nTregs), show a stable expression of Foxp3, but this property was shown to be due to additional mechanisms at the epigenetic level [64, 65].

The interplay between iTreg and Th17 intracellular differentiation networks is shown in (Figure 1.12).

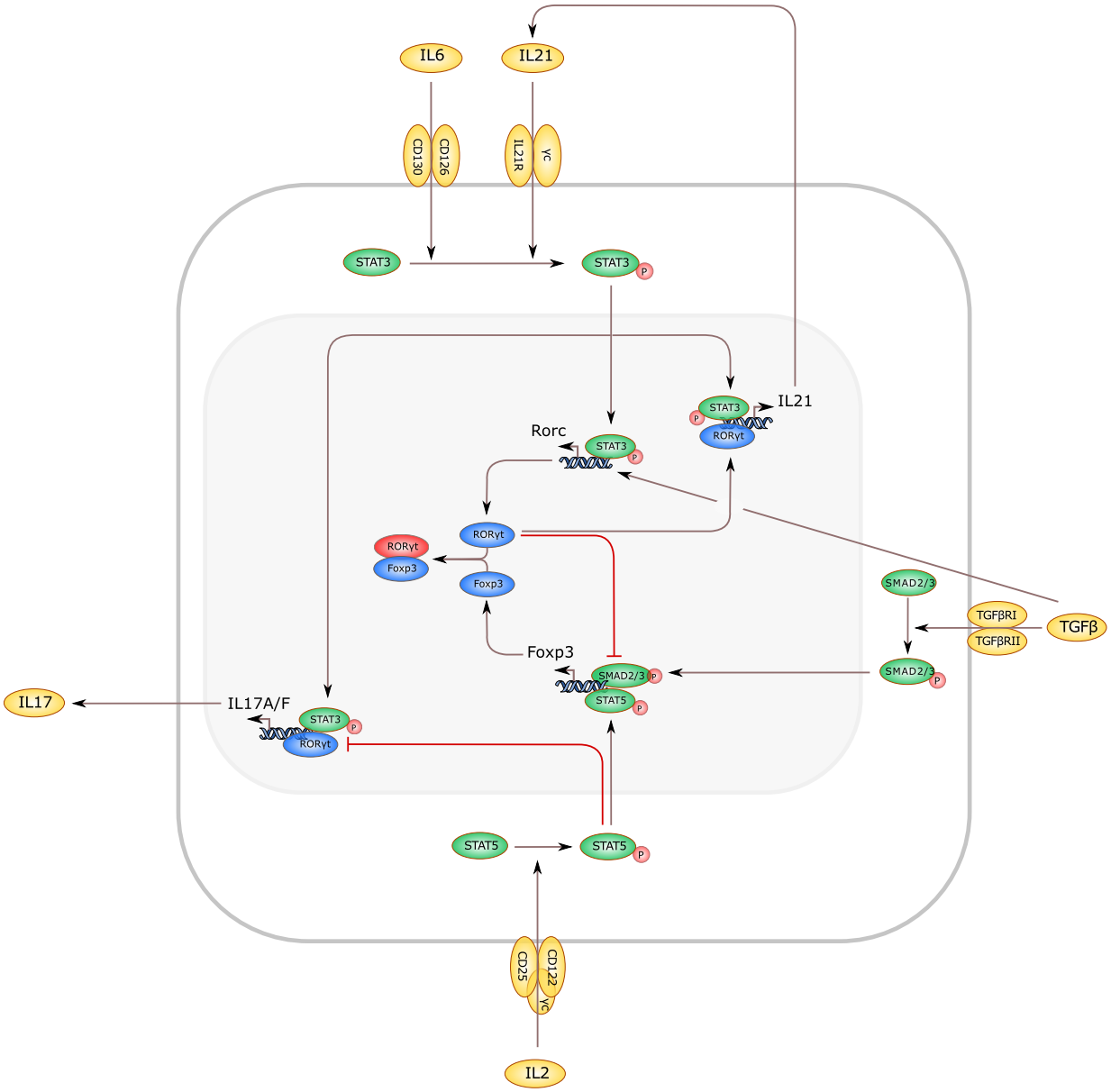


Figure 1.12: **Th17 - iTreg differentiation network.** TGF-β initiates the transcription of both Foxp3 and Rorγt, while IL-6 signaling activates both Rorγt and IL-21 expression. IL-21, in turn, sustains Rorγt via the same signaling pathway than IL-6. IL-2 support Foxp3 expression while inhibiting IL-17 expression. Foxp3 inactivates Rorγt by direct binding while Rorγt inhibits Foxp3 transcription.

1.6 Mathematical models for T helper differentiation

Several quantitative mathematical models have been developed to account for T helper differentiation, at different scales. Most of them are combined with the design of experimental data to train the model and to obtain realistic parameter through parameter estimation. They rely on a simplified regulatory sub-network, and mostly focus on a subset separately.

Models for intracellular signaling during Th1 differentiation IL-12 signaling during Th1 differentiation has been modelled in [161]. The kinetics of expression og IL-12Rβ2 were followed in vitro after adding IL-12 or not at day 2 following activation, and a mathematical model was designed to mimic/modelize the kinetics of the intracellular signaling (Receptor dimerization, STAT phosphorylation). Experimental data were used to discriminate the relative importance of redundant pathways up-regulating IL-12Rβ2. This model predicted that, at 3 days post activation, STAT4-P poorly induces IL-12Rβ2, which is already expressed at high rate (probably due to TCR activation), and that the effect of SOCS1 is very limited.

This model was extended by looking at the cross-talk between IL-12 and TNF-α on a Th1 cell line (2D6 cells) [162]. The cells were treated with IL-12 after 12 hours of culture, and the kinetics of IFN-γ, TNF-α secretion and IL-12R and STAT4-P expression were measured. In silico, STAT1 signaling and TNF-α production were added to the model, and a fitting procedure allowed to predict that: 1/ the effect of STAT4-P in sustaining TNF-α levels is negligible (TNFα is amplifying itself), 2/ that cell division induces a substantial decrease of STAT4-P under the hypothesis that it is not re-synthesized, 3/ that STAT4-P is able to induce IFN-γ at low dose while it requires a higher level of STAT4-P to induce IL-10, and 4/ that IL-12R phosphorylates both STAT1 and STAT4.

In [163], a model was developed to recapitulate IFN-γ and IL-4 signaling and the negative feedbacks of SOCS proteins on differentiation. A very detailed mathematical model was derived (>200 parameters) but due to lack of quantitative data, a set of parameters had to be chosen, that reproduces qualitatively the properties of differentiation under different doses of IL-4 and IFN-γ. The model predicted quite intuitive results, such that SOCS1-/- mice would have a skewed differentiation towards Th1, or that the use of JAK3 inhibitors would reduce Th2 differentiation. This study is more a 'proof of concept' of what you would expect was consistent with quantitative simulations, performed on a complex network.

The cross-talks between TCR, IL-12 and IFN-γ during Th1 differentiation were investi-

gated in [111] using WT or IFN-γR-/- T cells. The authors observed that, under Th1 condition, the first peak of T-bet expression at 24 hours following activation requires IFN-γ while the progressive up-regulation of T-bet at later time points relies on STAT4-P and is independent of IFN-γ, therefore segregating over time the IFN-γ and IL-12Rβ2 positive feedback loops. In silico, four different sub-networks were proposed to explain the regulation of T-bet and IL-12Rβ2 expression over time. The 4 models differ in including (IL-12 activates T-bet) or not, and including (TCR inhibits IL-12Rβ2) or not. The models without negative feedback were discarded because they could not explain a two-peaks temporal profile for T-bet, and the best model according to the data was the one where TCR inhibits IL-12R expression and where IL-12 signaling contributes to T-bet induction. While the remaining model shows the best consistency as compared to the 3 others ones, it is not possible to conclude for sure that this mechanism (TCR inhibits IL-12Rβ2) is the reason for T-bet downregulation after 24 hours, because this is the only inhibitory mechanism that was investigated among a longer possible list of described mechanisms in literature. Probably, comparing more models with different possible inhibitions would hihglight multiple mechanisms. As experimental validation, they show that blocking TCR signaling by inhibiting Calcineurin at day 1 increases T-bet and IL-12Rβ2 expression at later time points. However, a recent study suggests that TCR signaling has a positive effect on IL-12Rβ2 expression, and in a dose-dependent manner [164]. It would mean that the in silico model managed to reproduce the accurate curves for T-bet, but by using an explicit mechanism that likely does not exist.

Models for Th1-Th2 differentiation At the population level, a first model [165], further extended in [166], investigated the general properties of Th1 and Th2 cross-inhibition. It uses a simple topology of the network where Th1 and Th2 differentiation programs auto-activate themselves and inhibit each-other, through the production of generic Th1 and Th2 signals without explicitly naming which ones. The model is not compared with experimental data but instead characterizes the outcome of differentiation for every possible value of the unknown parameters. It predicts among other things that 1/ a mixed population of Th1 and Th2 is possible by combining both Th1 and Th2-inducing signals, and 2/ that re-programming using the other cytokine signal is possible. This prediction is not true in all contexts because Th1 or Th2 cells fully differentiated in vitro with multiple rounds of polarization are resistant to reprogramming in vitro, suggesting for additional layers or signals of regulation of the Th1/Th2 balance [84].

In [87], the intracellular levels of IL-4, IFN-γ, T-bet and Gata3 were measured by Flow

Cytometry, when T cells were activated under different combinations of IFN-γ and IL-4 doses. Double positive T-bet+Gata3+ cells could be observed when both signals were present. A simple mathematical model was designed, including (indirect) auto-amplification of T-bet and Gata3, and cross-inhibitions between them, similarly to [165], and could recapitulate the data. It shows that such a simple mechanistic approach can account for the cross-talk between IFN-γ and IL-4.

In [167], one week old Th2 cells were restimulated with PMA/Ionomycin and the kinetics of IL-4 expression at mRNA, protein and secreted protein (by IL-4 secretion assay) levels were followed. The cells showed a "yes-or-no" expression pattern. In order to understand the population dynamics of IL-4 expression, an in silico model of chromatin opening of the IL-4 locus was developed at a single-cell level, where the locus is initially in a 'ON' state and takes time to close to a 'OFF' state. During the 'closing', it has a higher chance to re-activate itself. Interestingly, 3 weeks-old Th2 cells produced less IL-4 after restimulation, which could be explained in the model by only changing the chromatin opening rate in differentiated cells. The prediction is therefore that the IL-4 locus is harder to reopen in more differentiated (older) cells. The model also allowed to extract the half life and transcription rates of Gata3 and IL-4 from the experimental data.

Models for Th17-Treg differentiation In [168] the effects of the topology of three different simple networks for Th17/iTreg differentiation were compared. All of them predicted the existence of a Rorγt+Foxp3+ state, and a possible reprogramming from iTreg to Th17.

In [169], the genome-wide expression of genes was followed by microarrays along 18 time points during the three first days of Th17 and Th0 differentiation. The ambition was to automatically reconstruct a genome-wide Th17 differentiation network. To this aim, potential interactions between genes were identified, provided the genes were differentially regulated compared to the Th0 control condition, and provided they were timely consistent with an activation. For instance, if a gene was expressed before another one, it could be an activator of it. This strategy highly over-estimates the number of interactions, and requires additional steps to identify the relevant ones. The expression of 39 different genes was perturbed by small interfering RNAs from the beginning of differentiation to reveal functional interactions between genes. At 48hrs, the impact of each perturbation was quantified and summarized by positive or negative arrows. As a limitation, since they look at the outcome 48hrs after the perturbation, the interactions might be indirect and the number of arrows is likely to be overestimated again. Among the newly identified factors impacting Th17 dif-

ferentiation, they further validated experimentally the impact of the deficiency of 4 of them on Th17 differentiation. To sum up, even if this study generated an enormous amount of experimental information and found new regulators, it was not sufficient to have a sufficiently constrained network. Additionally, the resolution of the network was limited to the topology of interactions between genes, and did not allow to perform kinetic simulations because the quantitative properties of the interactions are unknown.

In [109], the biological question was the same but the utilized methods were slightly different. They focused on the role of IRF4, BATF, STAT3, cMAF and Rorγt on Th17 differentiation. From a Chip-Seq approach, they identified the binding sites of combinations of these factors. BATF and IRF4 were required for most of the binding sites. Then, following gene expression by RNA-seq of Th17 generated from deficient mice in each of these genes, they could map positive or negative effects regarding these 5 transcription factors on the genome. Their data also pointed out potential regulators of Th17 and they experimentally assessed the impact of the upregulation or inhibition of several factors by siRNAs on Th17 differentiation at the transcriptomic level. Some of them had a critical effect, particularly FOSL2, and they proposed a model to add FOSL2 effect on the core ' IRF4, BATF, STAT3, cMAF and RORC' network. The focus was put on the structure of the network (who activates or inhibits who), and the kinetic properties of the system were not investigated.

Models for all subsets A long series of T helper differentiation boolean models were published from the teams of Denis Thieffry (ENS Paris) and Luis Mendoza (Instituto de Investigaciones Biomedicas, Mexico). Two recent studies were published in [170] and [171]. They designed complex networks from the literature on T helper differentiation (> 40 genes !), and simulated them in a boolean way (genes are only ON or OFF at any time). They could predict the stable states of the system (that would correspond to T helper subsets), including mixed states (with multiple phenotypes at the same time). However, boolean networks are not able to reproduce the kinetics of gene expression, and they don't have realistic timescales. Additionally, the rules to update gene expression at the next time-step can become very complicated. For instance, the decision of the next state of a gene regulated by an activator and an inhibitor when they are both 'ON' is an hypothesis to be chosen. When more genes are included, the list of regulation hypothesis to consider grows exponentially. New techniques using model-checking allow to incorporate experimental data as qualitative constraints [172] and to predict properties of the differentiation network with a minimum of user-defined hypotheses. It has been successfully applied to the T cell differentiation

network in [173], and predicted possible trajectories (re-differentiation) between differentiated T helper subsets.

Only one dynamic model for T helper differentiation including 4 T helper subsets (Th1, Th2, Th17 and iTreg) has been published so far [174]. It contains the cytokine signaling pathways and the main cross-inhibitions between transcription factors (Gata3, T-bet, Rorγt and Foxp3), including the regulation of cytokine signaling by SOCS proteins. The number of unknown parameters is very high (around 300), and the experimental data limited to only one late time-point. The model was designed to reproduce steady-state (late) expression of the genes, but has not been trained to reproduce the dynamics of gene expression during differentiation, which is the focus of the present thesis.

Published datasets that could be used for developing a T helper differentiation model
Several quantitative and genome-wide datasets following T helper differentiation in vitro have been published and could be the experimental basis of a new mathematical model :

- in [109], the genome-wide kinetics mRNA expression of Th17 in vitro differentiation were followed by the use of RNA-seq. As a control, a Th0 condition with anti-IFNγ and anti-IL-4 was followed. This dataset was very helpful to validate our kinetics in the Th17 condition, and correlated nicely (shown in Appendix, Figure 7.5).

- a similar trancriptomic assay, performed with microarrays, followed Th17 differentiation in vitro, at 18 different time-points [175]. The dataset was not accessible without using specific bio-informatic tools, and I gave up to open them.

- a kinetics analysis following Th9 differentiation was performed by [176]. The dataset is actually not publicly available and the authors didn't reply to our request to share their data.

- in [177], a transcriptome study following differentiating Th1 and Th2 cells was published. The expression of selected differentially expressed genes is given and rounded (with 10% precision). Some of the main factors of the differentiation network are not given in the list, and the authors didn't reply to our request to provide the full dataset.

- A study followed early Th1-Th2 differentiation from human cells in vitro, and shared publically the transcriptomic data [178]. Unfortunately, this was performed on human cells, and does not contain enough time-points to delineate clear kinetics, but the data was informative to compare with our own kinetics.

As a conclusion, even if genome-wide studies have been performed and gave important insight into the properties of T helper differentiation, these dataset were not made available in a manner that is suitable for later modeling, or for being compared with each-other, and I believe this is unfortunately a recurrent problem faced by the mathematical biology community.

As a second approach, the kinetics of the main factors could be taken from different studies separately. As an example, different kinetics have been published for T-bet mRNA expression during Th1 in vitro differentiation, in C57BL/6J mice, and with similar protocols (Figure 1.13). The curves show inconsistencies in their peaks, and it is consequently very complicated and dangerous to use quantitative data from different studies and from different laboratories, because the variation is too strong.

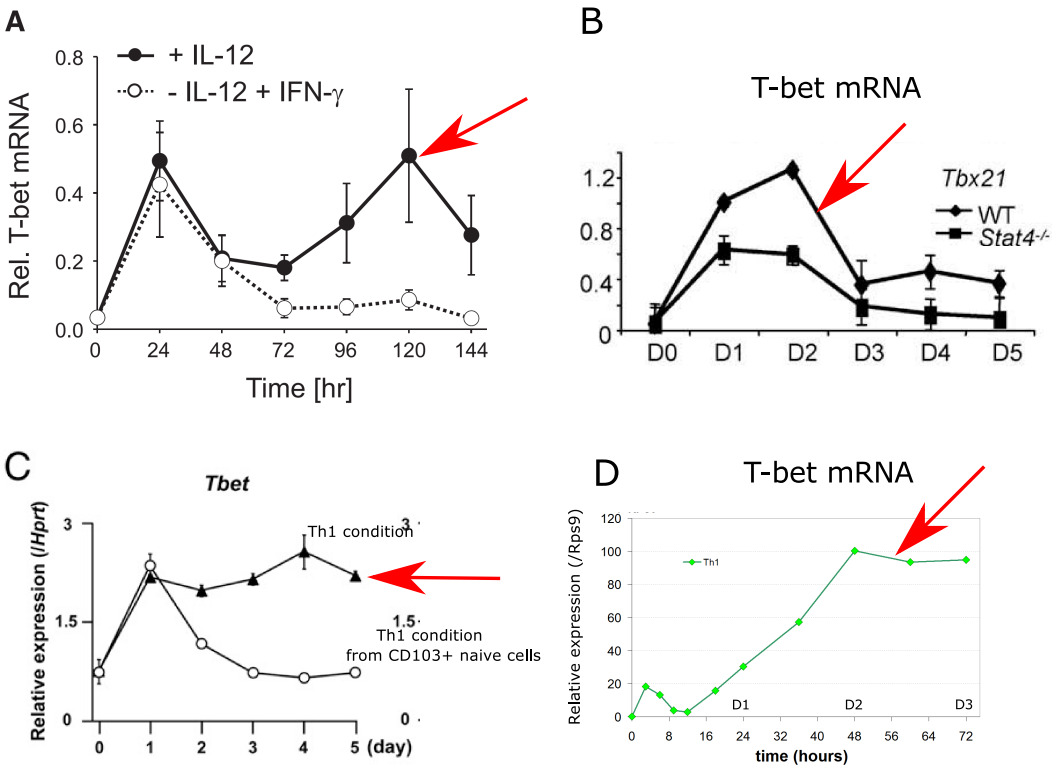


Figure 1.13: **Comparison of Tbet dynamics in Th1 conditions from different articles :** (A) only with IL-12 or under full Th1 conditions including IL-12 and anti-IL-4 (B,C,D). A : [111], B: [179], C: [180], D: from the present study. In theory, the curves pointed by the red should follow the same kinetics. Note that the curve in D. follows only 3 days of differentiation.

In our case, we decided to generate a complete data set (performed in the same experimental condition), in order to avoid these bias. I am very grateful that I had the opportunity to perform the experiments and time-points that were required for the project. I do hope that in the future, standardization of protocols will allow quantitative data to be compared between different studies, and that modellers will have more occasions to use published data without the necessity to generate new datasets.

CHAPTER 2

IMPACT OF GLUTAMINE DEPRIVATION ON T HELPER DIFFERENTIATION

2.1 Introduction

At steady state, naive T cells have not yet responded to an antigen and are small, quiescent, and possess a very small cytoplasmic volume (compared to the size of the nucleus). Upon activation, a slow growing phase is observed during which their volume increases, and the first division is finally observed 24 to 30 hours post-activation. In contrast, the subsequent divisions are performed with an extremely fast rate, up to three divisions per day [181]. The metabolic status of activated cells demands a metabolic reprogramming from their quiescent progenitors, and relies on the use of high amounts of energy and nutrients to build new constituents of the daughter cells, such as nucleic acids and proteins.

Naive T cells fulfil their energy requirements by the use of aerobic glycolysis of glucose (called 'Oxphos' glycolysis) and oxydation of fatty acids [182]. The other pathways are not shut down, but rather the balance between the different glycolytic pathways is skewed to the Oxphos one. Upon activation, a metabolic switch occurs [183], resulting in the upregulation of additional metabolic pathways, including 1/ anaerobic glycolysis pathways (called 'glycolytic pathway'), which use glucose faster but less efficiently than Oxphos, and generates lactate that acidifies the extracellular medium 2/ the pentose-phosphate pathway, that can convert glucose into ribose and nucleic acid precursors, and 3/ increased uptake of glutamine, as a source of both nitrogen for the generation of proteins, and of energy, by feeding the TCA cycle (Krebs cycle) [184, 185].

Th1, Th2 and Th17 cells harbor a high (non-Oxphos) glycolysis, and over-expression of

Glut-1, a major glucose transporter in T cells, was associated with an increased production of effector cytokines [186]. In Th1 condition in vitro, both proliferation and production of IFN-γ were reduced when glycolytic pathways were inhibited, while IL-2 production was not significantly impacted [187, 188]. Similarly, Glut1 deficiency selectively impaired the proliferation and differentiation of these T helper subsets [189]. In vitro activated T cells under low doses of glutamine show a reduction of proliferation and IFN-γ secretion when glucose concentration goes below 0.1 mM [190]. Such a low dose is likely not reached in most in vitro cultures with Fetal Calf Serum, which already contains glucose. Studies using glycolytic inhibitors such as 2-Deoxy-D-glucose, are sometimes used to mimic glucose deprivation, but should be interpreted carefully (reviewed in [191]), because of potential off-targets effects, including the blockage of the pentose-phosphate pathway which is necessary for glycosylation and which has been shown to impact on T cell differentiation [192].

iTregs, in contrast, rely more on the usage of fatty acids [193–195]. Blocking fatty acid oxydation by Etomoxir reduced the differentiation of murine iTregs in vitro [186]. Interestingly, the supplementation of fatty acids had an inhibitory effect on the survival of Th1, Th2 and Th17 and their cytokine production [186], meaning that not only deprivation but even excess of nutrients can impair certain differentiation programs. Finally, inhibiting glycolysis promoted murine iTreg differentiation in vitro [196].

The idea that iTregs rely on different metabolic sources than other subsets could be used therapeutically for restoring the balance between the pro- and anti-inflammatory CD4 T cell response. It would be further interesting to find metabolic differences distinguishing Th1, Th2 and Th17 differentiation programs.

While initial studies focused on the impact of glucose for T cell differentiation, little was known regarding the specific role of amino acids [197]. Notably, glutamine attracted our attention, as it is the most abundant amino acid in the plasma, and an important source of precursors for protein synthesis. Several studies have already pointed to the importance of glutamine: [198] compared the proliferation of T cells activated (Th0) in the absence of several identified amino-acids, and it resulted that glutamine was a limiting factor for T cell proliferation. The amount of IFN-γ and IL-2 were both reduced in the absence of glutamine. In [199], human PBMCs were activated with attenuated bacteria or measles virus and polarized in the presence of different doses of glutamine: in the absence of glutamine, Th1 differentiation was inhibited while the Th2 cytokine production was increased. In another study, human PBMCs were activated with anti CD3 with different levels of glutamine [190]. The early expression of mRNAs for IL-2, IL-4, IFN-γ were independent of the glutamine

concentration while the actual later IFN-γ secretion was reduced under limiting amount of glutamine. The production of other cytokines was not reported.

The work presented below was initiated following the observation that CD4 T cells activated (Th0 condition) under glutamine-deprived conditions resulted in a high percentage of Foxp3-expressing T cells. The aim of the present study was to characterize the impact of glutamine deprivation during in vitro differentiation of Th1, Th2, iTreg and Th17 subsets.

2.2 Material and Methods

Cell isolation and T helper differentiation C57BL/6J mice were purchased from Charles River laboratories. CD4 T cells were purified from freshly isolated lymph nodes and naive (gated as CD4+ CD62L+ CD44- CD25- cells) sorted by FACS. Cells were cultivated in glucose and glutamine-free RPMI 1640 medium (Life Technologies), complemented with 10% FCS, beta-mercapto-ethanol (50 μM), Penicillin Streptomycin, glucose (11mM), with or without glutamine (2 mM). Naive T cells were activated under polarizing conditions using non-(tissue treated) cell culture plates (Thermofisher), previously coated with 1μg/mL anti-CD3 (2C11, homemade) and 1μg/mL anti-CD28 (PV.1, BioXCell). After 3 days, cells were split and replated in cell-culture treated plates, and fed with 1 volume fresh media containing either IL-23 (to reach 20 ng/mL final) for the Th17 condition, or IL-2 (100 U/mL final) for the other conditions.

Notes: stopping TCR stimulation at day 3 avoids an excess of Activation Induced Cell Death (AICD). Anti-CD3 binds to a conserved part of the TCRs and leads to polyclonal activation (nonspecific) while anti-CD28 provides a costimulatory signal, by binding to CD28. It is an APC-free system, composed of only differentiating T cells and allowing to isolate RNA without the risk of contamination from other cell types.

Differentiation into T helper subsets was performed using: TH1 condition: IL-12 (10 ng/ml) and anti–IL-4 antibody (5 μg/ml); TH2 condition: IL-4 (10 ng/ml) and anti–IFN-γ antibody (10 μg/ml); iTreg condition: human TGF- b (3 ng/ml) and IL-2 (100 U/mL); Th17 condition: IL-6 (30 ng/mL), human TGF-β (3 ng/mL), anti-IFN-γ (10 μg/mL), and anti-IL-4 (5 μg/mL). Following isolation, naive T cells were washed 2 times with PBS, labelled with Cell Trace Violet (Invitrogen), 5 μM, for 3 minutes at 37°C, and resuspended in fresh medium with FCS, prior to activation.

Flow cytometry Flow cytometric assays were performed on Facs Canto II (BD Biosciences). At the time-point of interest, cells were stained for extracellular markers (CD4-AlexaFluor780,

BD), and intracellular stainings were performed using the eBioscience intracellular staining kit. Foxp3-PE and Foxp3-PeCy7 (eBioscience) were used. For intracellular cytokine staining, cells were washed and resuspended in RPMI containing glucose (11mM) and glutamine (2 mM), and supplemented with Phorbol Myristate Acetate (100 ng/mL, Sigma-Aldrich), ionomycin (1 mg/mL, Sigma-Aldrich) and brefeldin A (10 mg/mL, Sigma-Aldrich) for 4 hours at 37°C prior to intracellular staining. IL17A-PE and IFN-γ-APC were used (eBioscience). Analyses were performed on FlowJo (Tree Star, Inc.). Cytokines released in the supernatant were measured using the Cytometric Bead Array assay from BD, according to manufacturer protocol.

RT-qPCR RNA isolation was performed using RNeasy Micro Kit (Qiagen), Reverse transcription using the QuantiTect Reverse Transription Kit (Qiagen) and the q-PCR step was performed with LightCycler 480 SYBR Green I Master kit (Roche). The furnisher's protocols were followed. The primers sequences utilized are:

T-bet	S:5'-TCCCCCAAGCAGTTGACAGT	AS:5'-CAACAACCCCTTTGCCAAAG
Gata3	S:5'-AGTTCGCGCAGGATGTCC	AS:5'-AGAACCGGCCCCTTATCAA
Hprt	S:5'-CTGGTGAAAAGGACCTCTCG	AS:5'-TGAAGTACTCATTATAGTCAAGGGCA

The list of reagents is provided in Table 2.1.

2.3 Results

Impact of glutamine deprivation on T cell growth and proliferation during T helper polarization. To evaluate the importance of glutamine during polarization, naive T cells were differentiated in vitro towards a Th1, Th2, iTreg and Th17 fate (and 'Th0' as a control without polarizing cytokine), under glutamine-replete or -deprived conditions.

Cell growth was followed by flow cytometry (size monitored by Forward light, FSC and granularity by Scattered light SSC) (Figure 2.1). At 24 hours, T cells activated under glutamine-deprived conditions were still very small as compared to the cells activated in glutamine-replete conditions. However, the overall viability and the percentage of live cells was similar between conditions at this early time point. At a later time point (7 days), the cell size of T cells deprived of glutamine was similar to the one of cells activated with 2mM glutamine. However, it's important to note that the viability and growth of Th17-polarized cells was severely impaired under limited conditions. Therefore, the absence of glutamine is delaying, but not impairing the growth of Th1, Th2 and iTreg cells. In both glutamine

Table 2.1: List of reagents for Chapter 2

Reagent	Furnisher	Reference
CD4+ enrichment		
CD4 T-cell isolation kit, mouse	Miltenyi Biotech	130-095-248
LS columns	Miltenyi Biotech	130-042-401
Sorting		
FcBlock (2.4 G2)	BioXCell	BE008
CD4-Pe	BD	553049
CD8-PerCP	BD	551162
CD62L-FITC	BD	553585
CD25-AAF	eBioscience	47-0251-82
CD44-PeCy7	eBioscience	25-0441-82
DAPI		
Cell Culture		
untreated plates 24 wells	D Dutscher	55620
Fetal Bovine Serum	Eurobio	S1830-500
RPMI w/o Gln w/o Glc	D Dutscher	P04-17550
P/S	Life Technology	15140122
anti-CD28 (PV1)	BioXCell	BE0015,5
anti-CD3 (2C11)	homemade	
Cell Trace Violet	Life technology	C34557
mIL12	Peprotech	210-12-13
mIL2	Peprotech	212-12-20
mIL6	Peprotech	216-16
huTGFb1	R&D	240.B.002
mIL23	R&D	1887.ML.010
mIL4	R&D	404.ML.010
anti-IL4 (11B11)	BioXCell	BE0045
anti-IFNg (XMG1.2)	BioXCell	BE0055
mRNA		
RNeasy Mini Kit (250)	Quiagen	74106
QuantiTect RT (200)	Quiagen	205313
LightCycler480 SYBR green I	ROCHE	4707516001
Cytokine assay		
CBA mouse Th1 Th2 Th17	BD	560485
Stainings		
Foxp3 Staining buffer Kit	Ebioscience	00-5523-00
Fixation / Perm Kit	Ebioscience	00-5521-00
Foxp3-PE	Ebioscience	12-4774-42
Foxp3-PeCy7	Ebioscience	25-9985-80
CD4 AAF	BD	470042
mIL17A-PE	Ebioscience	12-7177-81
mIFNg-APC	Ebioscience	17-7311-82
Brefeldin A (Penecil. brefeldian.)	Sigma	B7651
PMA	Sigma	P1585
Ionomycin (Strepto Conglo.)	Sigma	I9657

conditions, and at least at 3 days and at later tested time-points, Th1 cells seemed to reach a bigger size than the other polarized subsets It could be the consequence of asymmetric divisions previously described in Th1 cells, that would leave a bigger and smaller cell at each division [200].

In parallel, cell proliferation was monitored by following CTV dilution, a cytoplasmic fluorescent dye whose intensity gets diluted by cell division. The history of cell proliferation was assessed at day 2 and 6 upon differentiation (Figure 2.2).

At 48 hours post activation, the cells differentiated without glutamine still didn't start dividing (for all the polarizing conditions), while some of the cells differentiated in the presence of 2mM of glutamine already started proliferating, up to 3 divisions. After 6 days of differentiation, the cells cultivated with glutamine had divided at least 5 times. In contrast, under glutamine-deprived conditions, the number of cell divisions was strongly reduced. Additionally, as mentioned above, without glutamine, most Th17 cells didn't manage to divide and died (Figure 2.1), while only a small portion of the remaining cells could start dividing. Therefore, the cells stimulated under limited amount of glutamine started dividing with an important delay, and at a slower pace.

It shows that glutamine is a major source of energy for differentiating cells, and confirms previous findings that Th17 cells rely on the supply of amino acids to expand and differentiate more than other subsets [197], though the effect of glutamine was not assessed in this study.

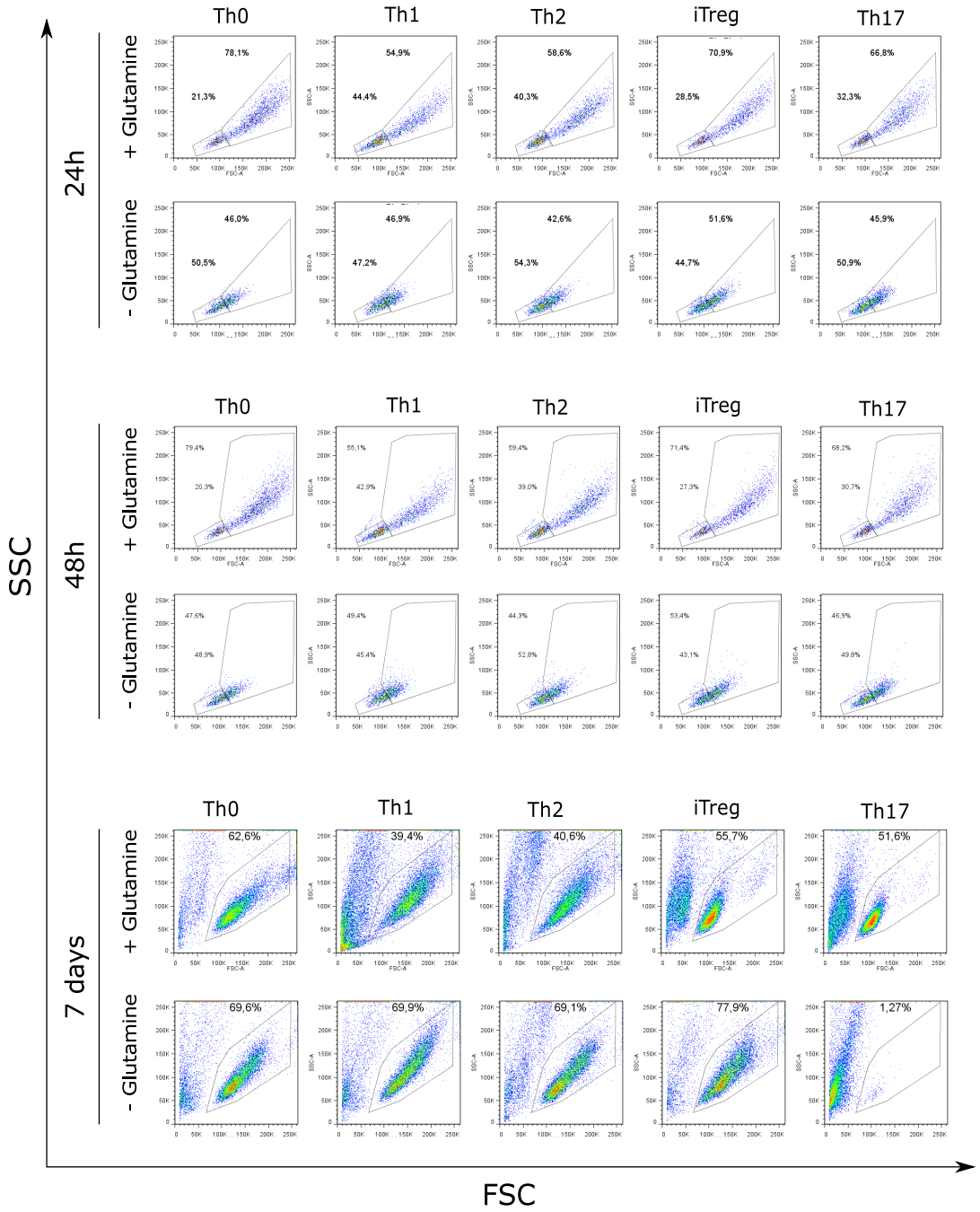


Figure 2.1: **Size (FSC) and Granularity (SSC) of cells after 24 hours, 48 hours and 7 days of differentiation**, under glutamine-replete or -deprived conditions. At 24 and 48 hours, the gates represent alive cells. 2 independent experiments. Note: The FSC scale is the same between 24 and 48 hours, but different for the day 7 time-point.

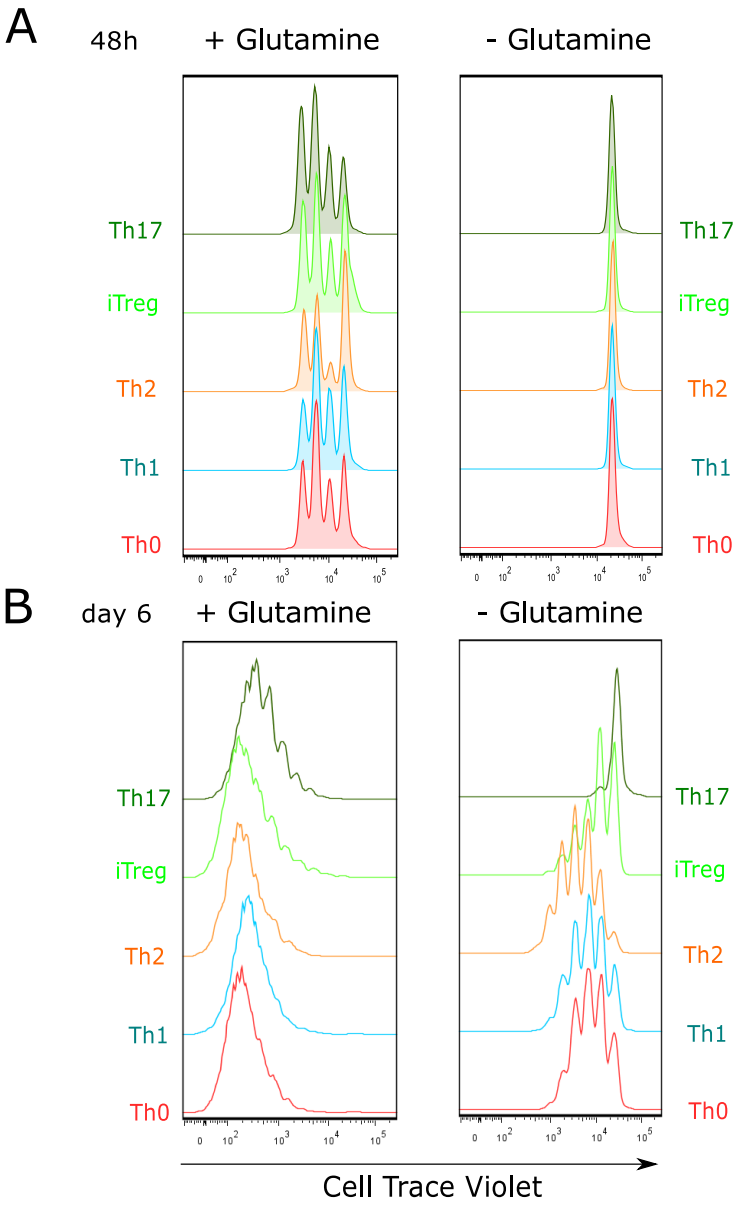


Figure 2.2: **Proliferation at day 2 (A.) and day 6 (B.)** of CTV-labelled T cells following T cell activation under polarizing conditions under glutamine-replete or -deprived conditions. (Representative of 3 independent experiments).

Glutamine deprivation impairs Th1 differentiation We further investigated whether specific differentiation programs were sensitive to glutamine deprivation. Therefore, at day 6 of differentiation, the production of effector cytokines was assessed following restimulation with PMA/Ionomycin under glutamine-replete conditions and intracellular staining for IL-17 and IFN-γ (Figure 2.3).

Under glutamine-replete condition, 57% of IFN-γ producers were detected in Th1 polarized cells. In contrast, only 4 percents of IL-17+ cells were detected in Th17-polarized cells. As expected, only few percents of cells activated under Th0 and iTregs conditions produced IFN-γ nor IL-17.

Interestingly, IFN-γ production was strongly impaired in Th1 condition under glutamine-deprived condition, falling from 57% to 5%, indicating the importance of glutamine to obtain an efficient Th1 differentiation. Without glutamine, the few remaining cells in the Th17 condition (due to massive cell death, see Figure 2.1), were not producing IL-17.

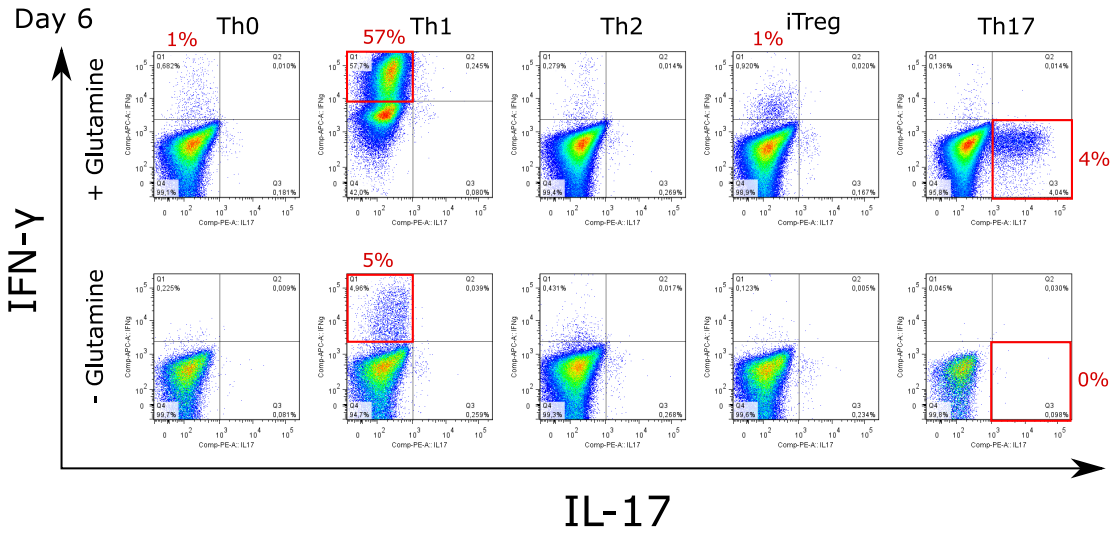


Figure 2.3: **Cytokine production following differentiation with or without glutamine.**Intracellular staining for cytokine production after PMA/Ionomycin restimulation at day 6. Representative of 3 different experiments.

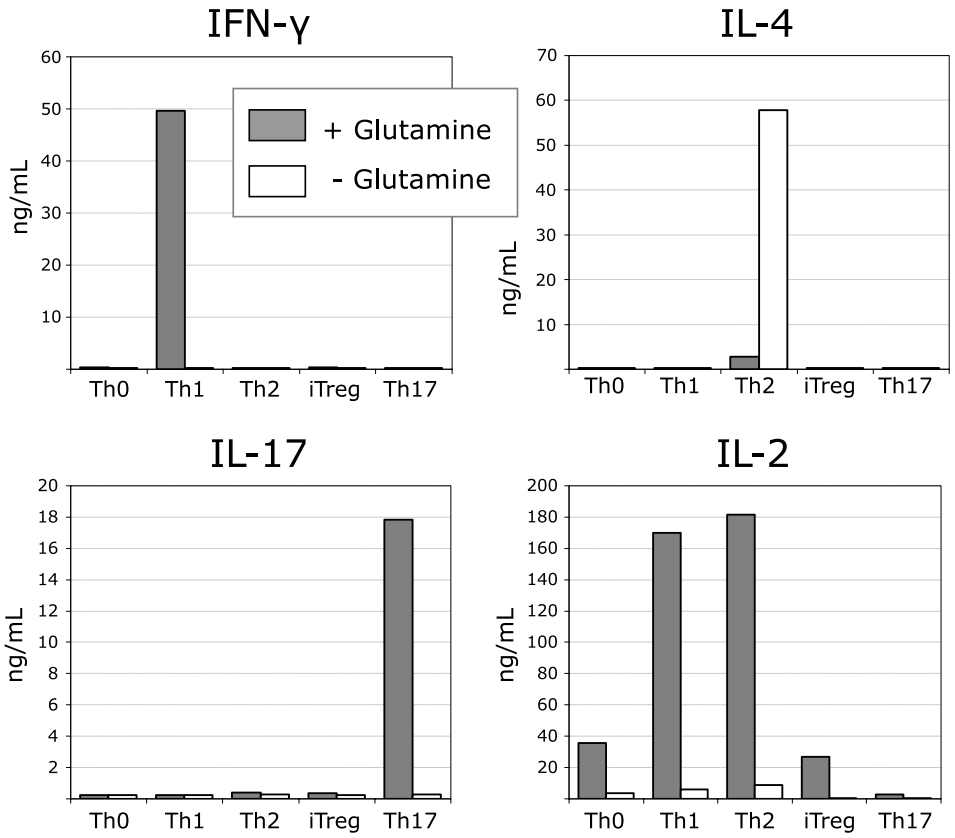


Figure 2.4: **Cytokine measurement in the supernatant of cultures at day 6 of differentiation,** in pg/mL, for each condition, with or without glutamine. (2 independent experiments).

In parallel, we also measured the concentration of specific cytokines in the culture supernatants of T cells activated under different polarizing conditions in the presence or not of glutamine (Figure 2.4). Consistent with intracellular stainings, IFN-γ production was strongly impaired in Th1 polarized cells under glutamine deprivation.

Interestingly, the IL-4 concentration in Th2-polarized cells increased in glutamine deprived condition, even though the density of cells was lower due to reduced proliferation, indicating that glutamine levels differentially modulate Th1 and Th2 differentiations, with opposite effects. As IL-4 was not detected in any other condition (and Th1 polarizing cultures are performed in the presence of blocking anti-IL-4), the impact of glutamine onto Th1 differentiation cannot be explained by a modulation of IL-4 production.

In normal conditions, the amount of IL-2 in the supernatant was higher in Th1 and Th2 conditions, reduced in the iTreg condition, and poorly detected in the Th17 condition most probably due to the presence of anti-IL-2. The amount of IL-2 in the supernatant results from a balance between cytokine production and consumption by binding to the IL-2 receptor complex [201], and makes the interpretation of these results tricky. For instance, the reduced amounts of IL-2 in the iTreg condition could be consistent with both a reduced production of IL-2 or a higher consumbtion. Nevertheless, under glutamine deprivation, IL-2 secretion was strongly abrogated in all conditions, consistently with [198], and possibly esplaining the reduced proliferation.

Note: Here, a low number of IL17+ cells was detected in the Th17 polarizing condition (4% under glutamine-replete conditions, and 18 ng/mL detected in the supernatant). It can either be explained by a non-optimized staining protocol or from a suboptimal protocol of polarization. In chapter 3, the protocol was modified using a different medium with an optimized combination of cytokines that allowed to reach 60% of IL-17 producing cells and more than 200 ng/mL in the supernatant.

Glutamine deprivation increases the generation of Foxp3 expressing cells The impact of glutamine deprivation on iTreg differentiation was then investigated. With this purpose, the percentage of Foxp3-expressing cells was assessed at day 3 and 6 of in vitro polarization (Figure 2.5).

At day 3, as expected, a high percentage of Foxp3+ cells was observed in the iTreg condition, and interestingly, the efficiency of the polarization was not altered under glutamine-deprived condition. A lower and transient percentage of Foxp3+ cells was detected in Th17 condition, consistent with previous findings [202]. Finally and as expected, a negligible

percentage of Foxp3+ cells could be observed in Th0 condition.

Surprisingly, at a later time point following stimulation under glutamine deprivation, a high percentage of Foxp3-expressing cells (more than 30% of the cells) was detected in Th0 and Th1 cells but not Th2 cells .These data indicate that glutamine level is crucial to Th1-differentiated cells but not to Th2 fate, suggesting distinct nutrient requirements of different T helper subsets.

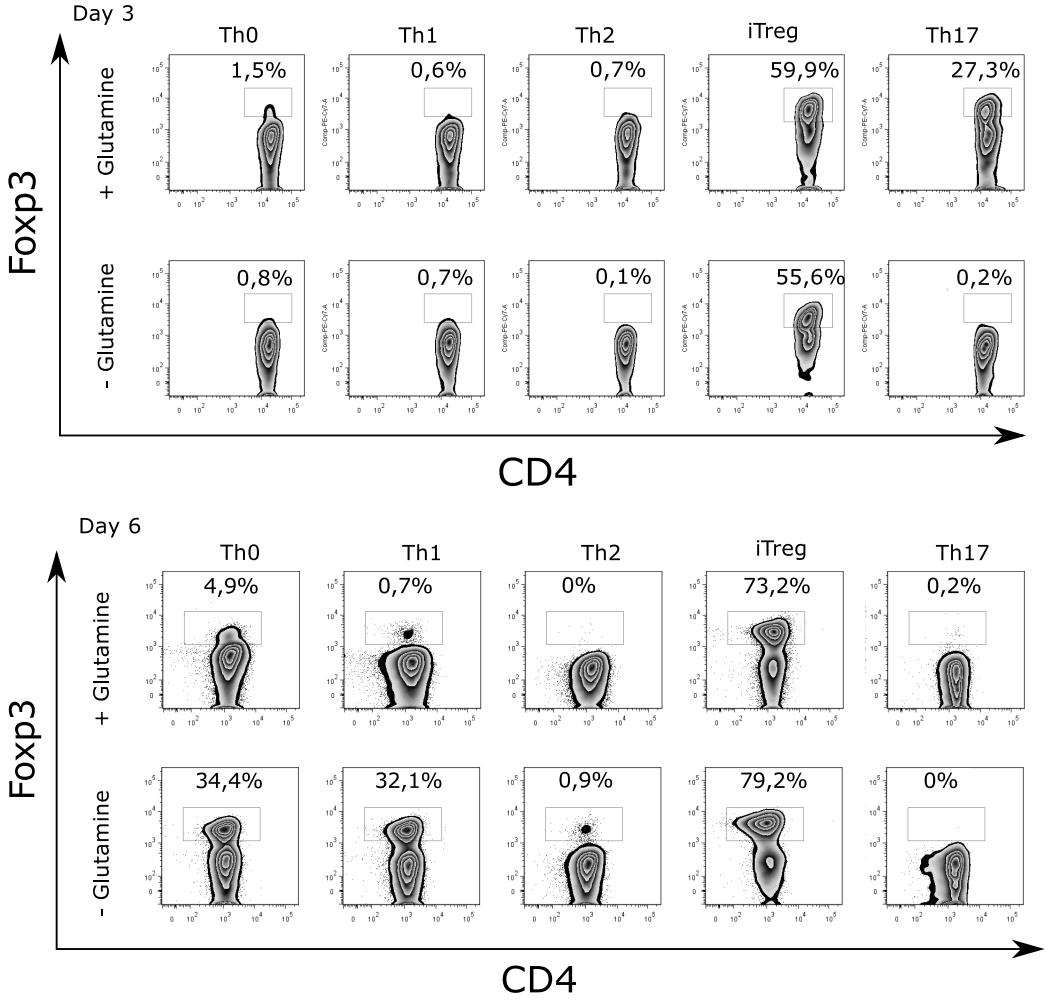


Figure 2.5: **Percentage of Foxp3-expressing cells following polarization in glutamine-replete or -deprived conditions.**Foxp3 intracellular staining was performed at day 3 and day 6 of differentiation. Note: CD4 expression level cannot be compared between day 3 and day 6 (not the same fluorophore). Representative of 3 independent experiments.

To understand why Th1 T cells could not be obtained under glutamine-deprived conditions, the level of T-bet and Gata3 mRNA expression were assessed at early time-points (Figure 2.6). Interestingly and consistently with the reduced IFN-γ production, T-bet mRNA levels were strongly reduced in Th1-polarized cells under glutamine-deprived condition, as compared to glutamine-replete condition. This low level of T-bet mRNA expression was further confirmed at the protein level (data not shown). In contrast, Gata3 mRNA levels were similar between Th2 cells polarized in glutamine-replete or -deprived conditions.

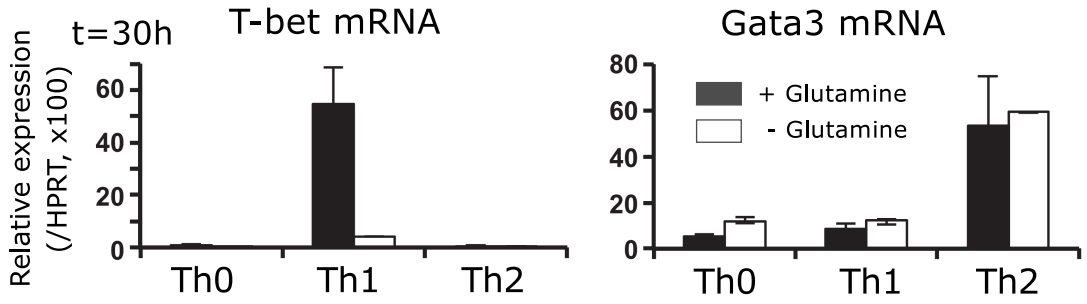


Figure 2.6: **T-bet and Gata3 mRNA expression**was assessed in T cells 30 hours following activation under Th0, Th1 and Th2 polarizing conditions, in glutamine-replete or -deprived conditions. Relative expression to HPRT is represented. 2 independent experiments.

Taken together, these results show that glutamine deprivation differentially impacts on the distinct T helper subsets: lower survival of cells polarized towards a Th17 fate; inhibition of Th1 differentiation, and conversion to Foxp3-expressing cells; increasing iTreg differentiation and promoting IL-4 production under Th2 condition.

2.4 Conclusions

In this chapter, we showed that glutamine deprivation has a critical impact on helper T cells differentiated in vitro. The proliferation and growth of activated T cells was considerably delayed, even though high amounts of glucose were present, showing that glutamine is a critical source of energy and signal for T cell differentiation.

It's important to mention that part of these results have been published in the following article: [203].

`Dorota Klysz et al. ‘‘Glutamine-dependent a-ketoglutarate production regulates the balance between T helper 1 cell and regulatory T cell generation’’. In: Sci. Signal 8 (2015)`

In line with this work, a study [204], evaluated the consequences of ASCT2 deficiency, a glutamine transporter, on mouse T cell differentiation. Consistently with our data, a de-

crease of Th1 and Th17 differentiations in vitro and in vivo was observed. Strikingly, no significant effect was revealed on Foxp3 in vitro (at day 4). Therefore, it would be interesting to further characterize the phenotype of these cells (other metabolic receptors for instance), and to activate them under low glutamine concentrations to determine whether ASCT2 was the main transporter responsible for glutamine uptake.

Recently, a study [205] confirmed our findings and demonstrated that glutamine deprivation promotes Foxp3 expression during human iTreg differentiation, and was associated with a preferential proliferation of Foxp3+ cells, outnumbering the Foxp3- cells with time.

To conclude this chapter, here, glutamine deprivation impacted the differentiation of all subsets. We opted for a mathematical approach to ultimately make predictions on which pathway is the most likely to drive the effect of glutamine.

CHAPTER 3

MODELING THE EARLY DYNAMICS OF T HELPER DIFFERENTIATION

3.1 Introduction

The mechanisms underlying T helper differentiation have been extensively studied, revealing large and complex networks linking differentiation signals to the expression of the master transcription factors or cytokines [206, 207]. However, due to their complexity, it is impossible to predict the behavior of T helper cells in new or pathological conditions just by drawing such networks, and a computational approach is required to assist our thinking.

As a first approach, several studies used a boolean network formalism, where each gene is either ON or OFF. They occurred to be powerful tools to study the topology of the T cell differentiation network and could predict potential new subsets or possible redifferentiation methods [208, 209]. However, predictions from boolean representations are limited by the lack of quantitation and dynamics: in these studies, all activations or inhibitions happen at the same time, and dose dependent interactions are not incorporated. New approaches are emerging using multi-valued logic or model checking [172, 173], and will likely improve the predictive power in a close future.

As a second strategy, dynamic models based on Ordinary Differential Equations are a suitable tool to simulate the kinetics of gene expression, in a manner that can directly be compared to experimental data [210]. They allow to incorporate more features into a differentiation network, especially the strengths and dynamics of gene interactions. Their major limitation is the number of generated parameters: each variable, activation and inhibition requires quantitative parameters, most of which are unknown. Therefore, a first reduction-

istic step is usually required, through the design of a minimal network based on the main genes and interactions. Based on experimental data, optimization methods further derive the most likely values for the parameters and the fitting quality between the model and the data gives a first insight on the trustability of the model.

While several dynamical models have been developed to describe the differentiation of one subset separately [111, 161, 167, 168, 211], or the balance between Th1 and Th2 fates [163, 166, 212], the differentiation of a naive cell into the main T helper subsets from the same network has still been poorly investigated, although it is necessary to understand plasticity between subsets. In [174], a large network was proposed, and allowed to recapitulate later stages of differentiation at steady-state. However, due to the lack of experimental data during differentiation, it can not be used directly to predict differentiation events nor plasticity at critical early time-points when the fate decision is taking place.

Here, we investigate the early dynamics of T helper cell differentiation in vitro at the mRNA and protein levels, in order to quantitatively assess the order of events underlying the fate decision, and to design an appropriate and minimal dynamical model capturing the key features of T helper differentiation. The kinetics of the major transcription factors and cytokines were measured during the first hours of differentiation into Th1, Th2, iTreg and Th17 cultures separately, including a Th0 control without added cytokines. Major delays could be identified at the transcriptional and post-transcriptional level, together with the low but significant expression of master transcription factors in their non-specific subsets, revealing the importance of inhibitions between the differentiation programs to keep the opponent factors under control.

We designed a minimal differentiation network from literature, including all the measured factors as mRNA and protein levels separately, to represent direct or indirect interactions between them. We used optimization methods to estimate the strength of the activations and inhibitions, with which the model successfully recapitulated all the measured data.

3.2 Materials and Methods, for chapters 3 and 4

Mice hCD2-Foxp3 Knock In mice, expressing a GPI-anchored human CD2-CD52 fusion protein under the control of Foxp3 promoter [213], were bred in-house (Helmholtz Centre for Infection Research, Braunschweig, Germany) under specific pathogen-free conditions and in accordance to institutional, state and federal guidelines.

Naive T Cell Isolation CD4+ T cells from lymph nodes and Spleen were purified with the MACS CD4+ T cell isolation kit (Miltenyi Biotec). Naive T cells were further sorted as CD4+ CD62L+ huCD2- cells with FACSAria (BD Biosciences) using CD62L-PercpCy5.5, CD4-V4-500 and huCD2-APC to leave the main color channels free for other stainings at early time-points. Cells were stained using Cell Trace Violet (Life Technologies) for 5 minutes at 37°C prior to culture.

T helper differentiation cultures. Naive T cells were activated on NUNC-Untreated plates (Thermofisher, ref 144530), pre-coated for 3 hours at 37°C with 1 μg/mL anti-CD3 (17A2, Biolegend), and 1 μg/mL anti-CD28 (37.51, eBiosciences). Cultures were performed on IMDM supplemented with 10% FCS, β-mercaptoethanol (50 nM), sodium-pyruvate (1 mM), hepes (30 mM), penicillin streptomycin (0.01 U), and glutamine (2 mM). The polarization conditions were as follow: Th0: medium only; Th1: 10 ng/mL IL-12 and 10 μg/mL anti-IL-4; Th2: 20 ng/mL IL-4 and 10 μg/mL anti-IFN-γ ; iTreg: 0.5 ng/mL TGF-β and 10 ng/mL IL-2 ; Th17: 30 ng/mL IL-6, 0.2 ng/mL porcine TGF-β, 10 ng/mL IL-1β, 7.5 μg/mL anti-IL-2 (JES6-1A12, Biolegend), 10 μg/mL anti-IFN-γ (XMG1.2, BioXCell). In experiments in which the inducing condition was changed during differentiation, the supernatant was carefully removed, and the cells were washed 2 times with PBS by gently adding it on top of the culture and removing it again. As the cells were sticking to the coated antibodies, they remained in the plate during the washing step, and kept receiving TCR stimulation.

Gene Expression and measuring cytokine levels At each indicated time-point, cells were centrifugated, and while the supernatant was taken to monitor cytokine levels, the pellet was resuspended in RLT buffer supplemented with β-mercaptoethanol and stored at -80°C. For each experiment, mRNA was isolated from all the samples at the same time using the RNeasy Micro Kit (Qiagen), and cDNA was obtained using the Transkriptor First Strand cDNA Synthesis Kit (Roche). To monitor gene expression, RT-qPCR analyses were performed using the LightCycler 480 SYBR Green I Master kit (Roche). The primers were designed to overlap an exon-exon junction with Primer Blast (NCBI), to avoid amplification of residual genomic DNA.

Gata3-Sense	AGAACCGGCCCCTTATCAA
Gata3-Antisense	AGTTCGCGCAGGATGTCC
Tbet-Sense	CACTAAGCAAGGACGGCGA
Tbet-Antisense	TCTGGGTGGACATATAAGCGG
Foxp3-Sense	CACACTTCATGCATCAGCTCTC
Foxp3-Antisense	GTGGGAAGGTGCAGAGTAGAG
Rorγ-Sense	TGCAAGACTCATCGACAAGGC
Rorγ-Antisense	AGCTTTTCCACATGTTGGCTG
IL-2-Sense	CAAGCAGGCCACAGAATTGAAA
IL-2-Antisense	AGAAAGTCCACCACAGTTGCT
IL-4-Sense	CAGCAACGAAGAACACCACAG
IL-4-Antisense	CAGTGATGTGGACTTGGACTCA
IL-10-Sense	GCCGGGAAGACAATAACTGC
IL-10-Antisense	GCCTGGGGCATCACTTCTAC
IL-17A-Sense	GGACTCTCCACCGCAATGAA
IL-17A-Antisense	CCAGCATCTTCTCGACCCTG
IL-21-Sense	TGAAAGCCTGTGGAAGTGCAAACC
IL-21-Antisense	AGCAGATTCATCACAGGACACCCA
TGF-β-Sense	GGAAGGACCTGGGTTGGAAG
TGF-β-Antisense	CCACGTAGTAGACGATGGGC
IFN-γ-Sense	CGCTACACACTGCATCTTGG
IFN-γ-Antisense	GTCACCATCCTTTTGCCAGT

The level of cytokines in the supernatant was measured by Flow Cytometry on a Fortessa (BD Biosciences), using the LEGENDplex™ Mouse Th Cytokine Panel (Biolegend). The standard curves were fitted to a 4-parameter log-sigmoidal function to retrieve the cytokine amounts from MFI to ng/mL.

Flow Cytometry Flow cytometric assays were performed on LSR Fortessa (BD Biosciences). Cells were stained with Life Dead fixable blue (Invitrogen) to exclude dead cells, prior to extracellular staining using huCD2-PeCy7 (Biolegend) for monitoring Foxp3 expression. Fixation and intracellular stainings were performed using the eBioscience intracellular staining kit. Gata3-A647 (BD), RORγt-PE (BD) and T-bet-PE (BD) were used. For intracellular cytokine staining, the cells were washed and stimulated in medium with Phorbol Myristate

Acetate (100 ng/ml, Sigma-Aldrich), ionomycin (1 mg/ml Sigma-Aldrich) and brefeldin A (10 mg/ml, Sigma-Aldrich) for 4 hours at 37°C prior to intracellular staining. Analyses were performed with FlowJo (Tree Star, Inc.).

Simulations and Parameter Estimation The program performing simulations and parameter estimation was developed in C++, using the Qt graphical library for plotting curves. The Ordinary Differential Equations (ODEs) (Box S9) were solved with adaptive Euler method with a minimum time-step and a stopping condition when one simulated variable exceeds a realistic range of values. It allowed to discard inaccurate parameter sets before the simulation ends, and avoided freezing in the case of steepness-inducing parameters sets. Due to the number of unknown parameters, the parameter estimation procedure was separated into sub-fittings [214]. Taking advantage of the availability of the kinetics at the mRNA and protein levels, and since the network doesn't have any direct feedback (mRNA impacting protein level impacting mRNA level and so on), each variable could be fitted separately with its subset of relevant parameters, by replacing all the other variables by a linearly interpolated curve from the data, following the method descrived in [214]. A final global fitting procedure was performed, 50% around the parameters values found from these step-by-step fitting (see Box S7). All optimizations were performed using the Evolutionary Strategy Algorithm described in [215], and further explained in (Box S1). 12 different mutations and 8 cross-over operators were compared for the fitting of a smaller regulatory network to synthetic data as benchmark. The best combination was SBX-cross over and normal mutation on all parameters. The 'sum of squares' cost function was used to compare a simulation and experimental data.

The reagents used throughout chapters 3 and 4 are listed in Table 3.1.

Table 3.1: List of reagents for Chapter 3 and 4

Reagent	Furnisher	Reference
CD4+ enrichment (on AutoMACS)		
anti-mouse CD4 (L3T4) MicroBeads	Miltenyi Biotech	130-049-201
Rat IgG, whole molecule	Dianova	012-000-003
FcBlock (α-CD16/CD32, 2.4G2)	BioXcell	BE0008
Erythrocyte lysis buffer	. 01M KHCO3, 0.155M NH4CL, 0.1mM EDTA	
Sorting		
CD4-V500 (RM4-5)	BD	560782
CD62L-PerCpCy5.5 (MEL-14)	ebioscience	45-0621-82
humanCD2-APC (RPA2.10)	BioLegend	300214
Cell Culture		
untreated plates 24 wells	Thermofisher	144530
α-CD3 (17A2)	Biolegend	100208
α-CD28 (37.51)	eBiosciences	12-0281-86
Fetal Bovine Serum	Biochrom	
IMDM + Glutamax	Gibko	21980032
HEPES-Buffer 1M	Biochrom	L1613
2-Mercaptoethanol, 50 mM (1000X)	Life Technologies	31350-010
mIL-12	Peprotech	210-12
mIL-4	R&D	404-ML/CF
porcine TGF?1	R&D	101-B1
mIL-2	R&D	402-ML
mIL-6	Biolegend	575204
mIL1b	Biolegend	575102
α-IFNγ (XMG1.2)	BioXCell	BE0055
α-IL-4 (11B1)	BioXCell	BE0045
α-IL-2 (JES6-1A12)	Biolegend	503704
mRNA		
RNeasy plus Mini kit (50)	Qiagen	74134
Transkriptor First Strand cDNA Kit	Roche	04379012001
Light Cycler480 SYBR Green I	Roche	04 887 352 001
Cytokine assay		
LEGENDplex Mouse (13) Th Cytokine Panel	BioLegend	740005
Stainings		
Cell Trace Violet proliferation kit	Invitrogen	C34557
Brefeldin A (Penecil. brefeldian.)	Sigma-Aldrich	B7651-5MG
PMA	Sigma-Aldrich	P1585-1MG
Ionomycin (Strepto Conglo.)	Sigma-Aldrich	I9657-1MG
Live/dead fixable blue (UV)	invitrogen	L23105
Foxp3 Staining Buffer Set	eBioscience	00-5523-00
Gata3-A647 (L50-823)	BD	560068
IFN gamma-FITC (XMG1.2)	BioLegend	505806
Tbet-PE (4B10)	BD	561265
IL17A-APC (TC11-18H10)	Biolegend	506916
IL4-PE (11B11)	Biolegend	504104
CD45.2-PerCP/Cy5.5 (104) Chap.4	BioLegend	109828
CD45.1-APC (30-F11) Chap.4	BioLegend	103112
Foxp3-PE (FJK-16S)	eBioscience	12-5773-82
human CD2-PeCy7 (TS1/8) ()	Biolegend	309214
ROR gammat-PE (AFKJS-9)	eBioscience	12-6981-82

Box S1 : Optimization method used. Optimizations are performed in a log scale, between the log of the boundaries values for each parameters. The algorithm follows an 'Evolutionary Strategy' design, where each individual I carries both a value ($I.p_k$) and a speed of mutation ($I.\sigma_k$), for each parameter/dimension k. The speed of mutation also evolves (mutates), in a way that some individuals are mutating faster (good explorers), while some individuals are mutating slowly (better exploitation). The selection process is therefore selecting on the parameter values, and indirectly on the speed of individuals.

Parameters:

PopSize	250	Population size
Number of generations	200 to 2000	Max number of simulations / PopSize
ForkCoeff	0.5	Birth rate : proportion of the population created at each generation
PrcOffspring	0.2	Proportion of newborns that come from cross-over (the rest come from mutations)
SigmasMut	0.005	Rate of changing the sigmas (mutation speed)

Algorithm 1 Optimization Algorithm (Evolutionary Strategy)

Initialize a population of PopSize random individuals (uniform for each parameter)
for each generation **do**
  Updating the Sigmas of the individuals, ($*N(0,$ mutation speed))
  for each individual I **do**
    for each parameter index k **do**
      if With probability SigmasMut **then**
        $I.\sigma_k$ $I.\sigma_k * exp(N(0,1))$ ▷ $N(0,1)$: normal centered gaussian
      end if
    end for
  end for
  AddOffspringByCrossOver($popSize.forkCoeff.PrcOffspring$)
  AddMutants(popSize . (1 - forkCoeff) . PrcOffspring)
  Selection:
  for each individual I **do**
    Simulate the ODEs with the parameters of this individual ($exp(I.\{p_1, p_2, ...\})$)
    Evaluate the cost ('Fitness') between the simulation and the experiments
  end for
  Sort all individuals based on their fitness, and keep only the 'PopSize' best ones (lowest fitness)
end for

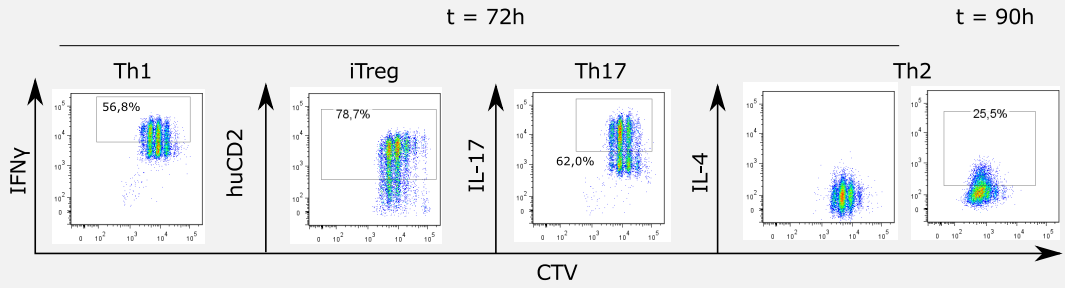
Note : Mutants for the parameter k are made according to a normal mutation, by adding $\sigma_k.sqrt(2).N(0,1)$. Offspring are made using the SBX cross-over, with parameter 1.0

3.3 Results

Three days of differentiation are sufficient for a fully established program. In order to define a time-window to monitor CD4 T cell differentiation, we performed in vitro cultures from naive CD4 T cells, activating in the presence of inducing cytokines for Th1 (IL-12 and anti-IL-4), Th2 (IL-4 and anti IFN-γ), iTreg (IL-2 and TGF-β), Th17 (IL-6, IL-1β, TGF-β, anti IL-2 and anti IFN-γ), and Th0 as control (no cytokine added).

The typical differentiation efficiency at day 3 was around 60% IL-17+ cells under Th17 condition, 60% IFN-γ high cells under Th1 condition, and 70-80% Foxp3+ cells in iTreg condition (Box S2). In Th2 condition, all the cells expressed Gata3 to high levels already at day 3, but IL-4 expression by cytokine staining was detected only later at day 4 when cells were replated in fresh medium at day 3, showing that the fate decision was already achieved at day 3 for all subsets. We therefore decided to restrict our study to the first three days of differentiation.

Box S2 : Efficiency of differentiation



Efficiency of differentiation, monitored by cytokine intracellular staining for IFN-γ, IL-4 and IL-17; and by expression of huCD2 (for Foxp3) after three days of differentiation. IL-4 intracellular staining is also shown at 90 hours (day 4) of culture, after replating the cells in a normal, non-coated culture plate with fresh medium and 10 ng/mL IL-2. Representative of 3 distinct experiments.

The kinetics of transcription factors expression reveals non-exclusive expression. The expression of the main transcription factors was followed during differentiation in the five conditions of interest: Th1, Th2, iTreg, Th17 and Th0 (Figure 3.1). mRNA levels were obtained from RT-qPCR while protein levels were monitored by flow cytometry. The transcription factors were significantly expressed in their associated subtype as early as 24 hours, but their expression was not limited to one subtype.

Indeed, T-bet showed a high but transient expression in Th0 and iTreg conditions, while it was only sustained in the Th1 condition. Similarly, Gata3 showed upregulation in Th0 and iTreg condition, consistent with findings that Tregs express Gata3 [102, 216] and T-bet [100] in vivo, while it was completely downregulated in Th17 and Th1. Finally, a transient expression of Rorγt mRNA could be observed in the iTreg condition, in accordance to the fact that TGF-β can induce both Rorγt and Foxp3.

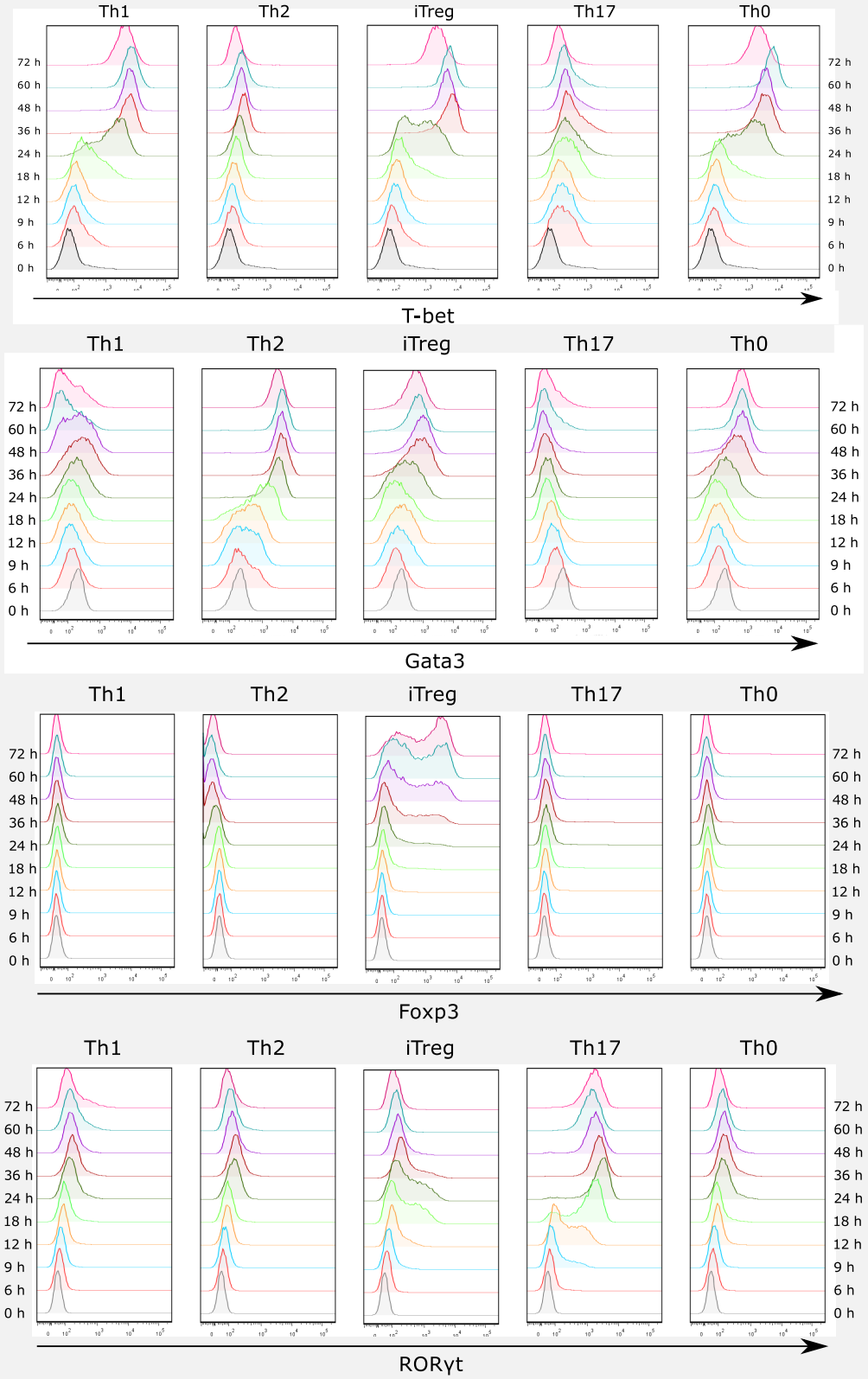
Surprisingly, an initial peak in T-bet mRNA level was observed in all subsets at the first time-point (3 hours), consistently with [109, 217], and without upregulation of the protein level before 16 hours, showing a limiting rate of T-bet translation at early times.

Gata3 mRNA and protein were already present in naive T cells, as already shown [217, 218] and linked with reminiscent expression of Gata3 from thymic development, but it was interesting to see a fast downregulation at both mRNA (as in [217]) and protein levels as early as 3 hours in all subsets but Th2. It could be explained by ubiquitinylation of Gata3 protein downstream TCR signaling [218, 219] that would further dampen Gata3 auto-activation [125]. A small peak of Gata3 mRNA could be observed in the iTreg condition, consistent with findings that IL-2 supports Th2 differentiation [220], and that STAT5 can bind to the gata3 promoter [221].

Rorγt peaked early, in both mRNA and protein levels around 18 and 30 hours, respectively. Both levels further went down to intermediate levels, even when differentiation was extended up to one week (not shown), while these cells are were still able to produce high amounts of IL-17 upon restimulation.

Finally, Foxp3 was expressed in an all-or-none manner (Box S3). The percent of Foxp3+ cells was following the amount of mRNA for Foxp3 found in the population. It is likely that the expression of Foxp3 mRNA also follows a yes or no pattern, but RT-qPCR could only monitor the population average. The details of the FACS stainings for each time points are shown in (Box S3).

Box S3 : Kinetics of Transcription factors levels by flow cytometry.



The histograms of fluorescence intensity are shown for each transcription factor at each time-point and each differentiation condition separately. A slight increase in fluorescence for T-bet and Rorγt was also observed for the isotype control stainings (not shown), and was deduced from the expression levels shown in (Figure 3.1). 2 distinct experiments.

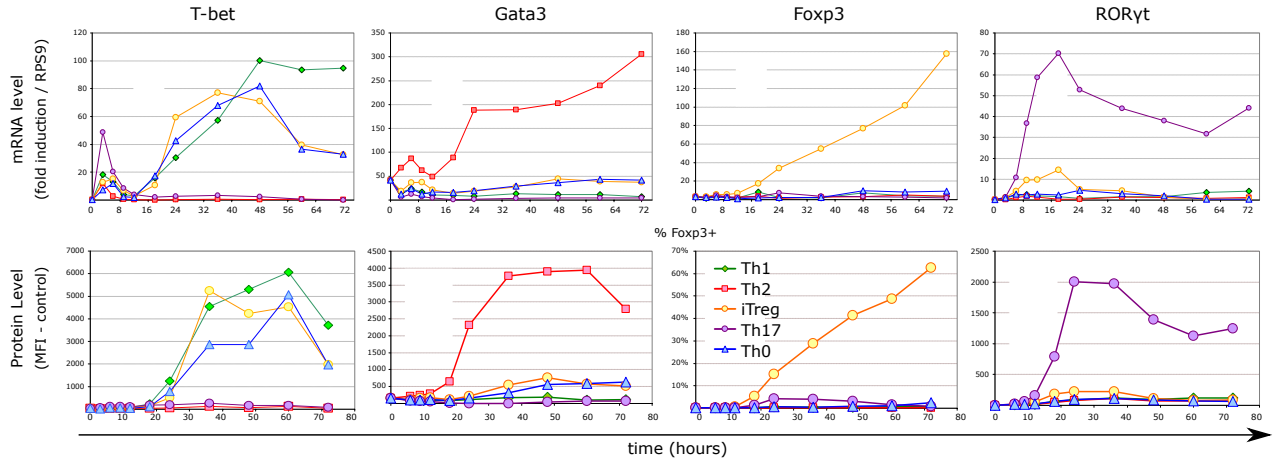


Figure 3.1: **Kinetics of master transcription factors during the first days of differentiation**, in Th0, Th1, Th2, iTreg and Th17 condition. A- Kinetics or mRNA expression, followed by RT-qPCR and normalized to RPS9 gene expression. B- Kinetics of protein expression followed by intracellular FACS staining (MFI sample - MFI isotype control). As Foxp3 expression always shows two populations with low and high Foxp3 levels, Foxp3 expression is represented as the percent of expressing cells. 2 different experiments.

Effector cytokine expression is specific but requires more time As transcriptions factors pop up early during differentiation, we wondered at which time cytokine feedbacks could already support or impact on transcription factor expression. We followed the dynamics of cytokine expression as mRNA levels by RT-qPCR and the amount of cytokines secreted in the supernatant (Figure 3.2). While the transcription factors were not exactly specific to T helper subsets, the expression of cytokines required more time and was more specific.

A very small peak of IFN-γ mRNA expression could be observed in all subsets in the first time-points (Box S4). Substantial amounts of IFN-γ mRNA appeared after 24 hours in Th1 condition and the cytokine could be detected in the supernatant at 36 hours.

IL-4 mRNA also showed a very small peak after activation (not shown), but was highly expressed only after 24 hours, with a similar dynamics than IFN-γ in Th1. As IL-4 is both the inducing cytokine used for Th2 differentiation, and produced as a feedback signal, the amount of IL-4 in the beginning is overriding the amounts of IL-4 produced during the first 72 hours. Consistently with the fact that IL-4 producing cells could be observed at day 4 (Box S2); in our experimental system, strong IL-4 production required more time than other subsets.

Finally, during Th17 differentiation, IL-21 mRNA was already detected after 6 hours, while the cytokine was only produced after 16 hours. As IL-21 is shown to enhance Rorγt and IL-17 expression, this supports the idea that IL-21 provides an early feedback loop during Th17 differentiation. It was noticeable that IL-21 was detected in the Th1 condition

as in [222, 223] but not as protein. It suggests that the translation or secretion of IL-21 is specifically repressed during Th1 differentiation.

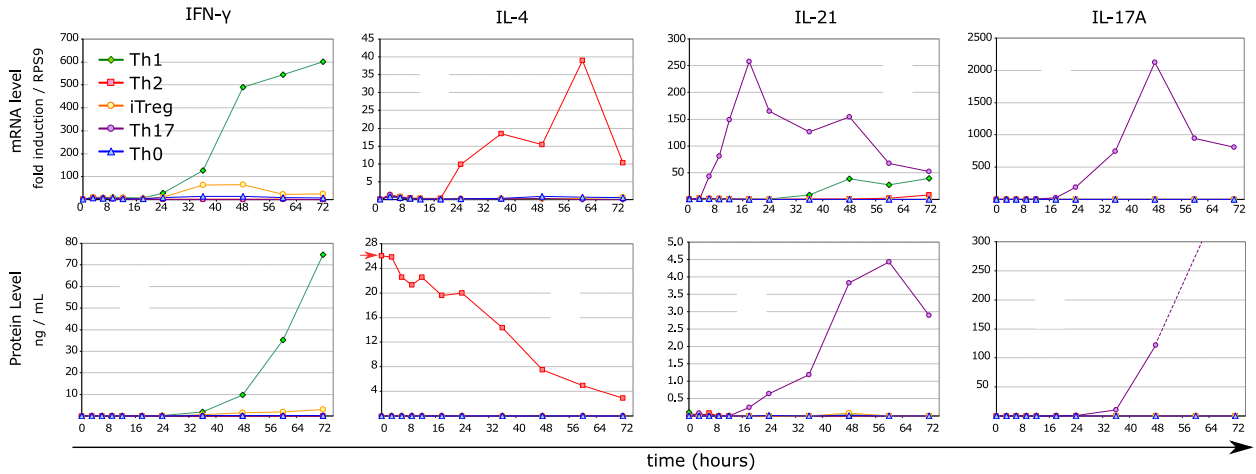
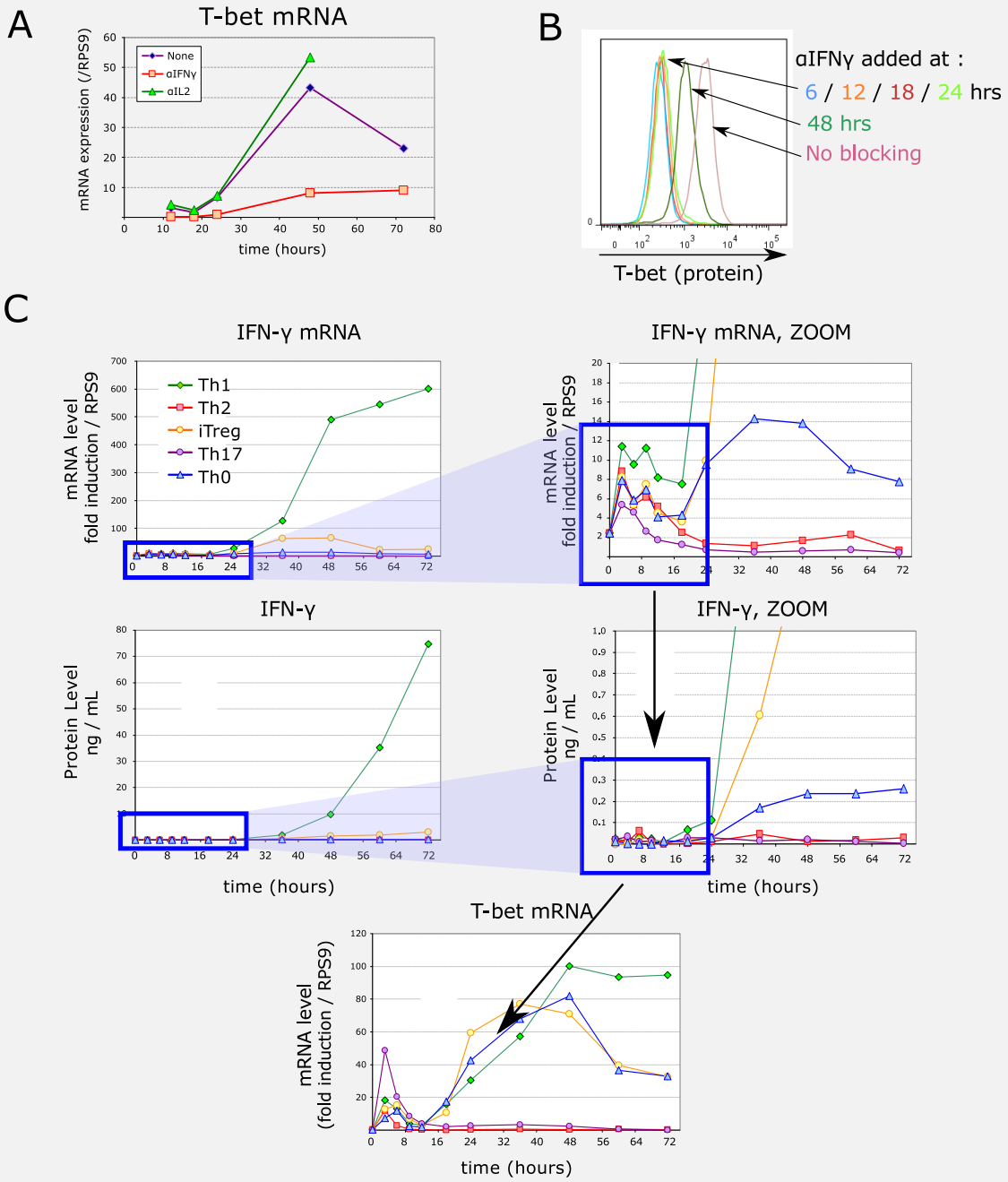


Figure 3.2: **Kinetics of cytokines expression during the first 3 days of differentiation**, in Th0, Th1, Th2, iTreg and Th17 condition. A - Kinetics or mRNA expression, followed by RT-qPCR and normalized to RPS9 gene expression. B - Cytokine levels in the culture supernatant, measured by multiplex (elisa protocol on beads, see Methods). Note that IL-4 is added to the culture from the start (red arrow). 2 different experiments.

Box S4 : Early undetectable levels of IFN-γ production are necessary for T-bet upregulation.



A: mRNA expression of T-bet, under the presence of anti-IL-2 or anti-IFN-γ. Blocking IFN-γ stops T-bet upregulation, even in the presence of IL-12 (Th1 condition) while blocking IL-2 does not impair T-bet expression. B: protein expression of T-bet monitored by flow cytometry at 72 hours, after adding anti-IFN-γ at different time-points. Blocking IFN-γ up to 24 hours abolishes T-bet upregulation at protein level at later time-points. C: Kinetics of IFN-γ expression at mRNA and protein level, with a zoomed view (right panel). Conclusion: IFN-γ cytokine in the medium are necessary for the upregulation of T-bet at 24 hours and later, though the presence of IFNγ was not detected by multiplex assay.

Non-specific cytokines are transcribed earlier but show delayed protein expression. Among cytokines produced by CD4 T cells, we followed the kinetics of other cytokines, not specific to particular T helper subset, but that could have an effect on differentiation (Figure 3.3).

IL-2 mRNA showed a first peak at 12 hours post activation in all subsets before going back to low levels at 72 hours, consistent with [224]. Two peaks could be observed in Th0, Th1 and Th2 conditions, suggesting that two different pathways control IL-2 expression over time. It was noteworthy that, in the presence of IL-2 in the iTreg condition, IL-2 expression was lower than other conditions, while in the presence of anti-IL-2 in Th17 condition, the early peak of IL-2 mRNA was higher, which is consistent with previous findings showing that IL-2 inhibits its own production [225, 226]. Interestingly, compared to the Th0 condition, Th2 cells produced less IL-2, supporting a negative effect of IL-4 to IL-2 production as well, as observed in [226].

The secretion of IL-2 in the supernatant showed a major delay compared to the expression of the mRNA in all subsets, and its upregulation could be detected only after 18 hours. Consumption of IL-2 by internalization by Tregs has been proposed as a suppressive mechanism [201]. In our system, the reduction of IL-2 in iTreg condition was minor, while the cells already expressed CD25 at 12 hours (not shown), supporting that this mechanism has a limited effect here.

IL-10 was detected in mRNA and protein level in Th1 and Th2 at day 3, while in Th17 condition, IL-10 came up earlier, but never reached the same level. IL-10 is usually described as a Treg and Th2 cytokine, but it has also been shown to be produced by Th1 cells under high TCR stimulation [195], which is likely the case with plate-bound stimulation. Regarding the iTreg condition, we couldn't detect IL-10. It is consistent with the fact that ex vivo Tregs do not express IL-10 in vitro, except if they are isolated from the gut [227, 228]. Additionally, iTregs can express IL-10 in the gut in vivo after adoptive transfer into mice with induced colitis [229].

Th17 cells were shown to produce IL-10 [78, 228], which has a regulatory effect on Th17 differentiation in vivo [230]. In order to find the impact of the presence of IL-10 in the supernatant on the differentiation profiles, we assessed the effect of blocking IL-10 in Th1, Th2 and Th17 cultures (Not shown). No impact could be detected at the transcription factor levels nor cytokine expression by intracellular staining. We couldn't either detect an effect of adding exogenous IL-10 in the cultures. It could be due to a lack of IL-10 receptor expression.

Finally, the kinetics of TGF-β mRNA showed a high peak after activation in all subsets, followed by a basal expression that was higher in iTreg and Th17 conditions. Activated T cells were already shown to produce TGF-β [142] in latent or activated form. However, due to the presence of high amounts of latent TGBβ in the serum, we couldn't measure the production of TGF-β in the supernatant by differentiating cells over time.

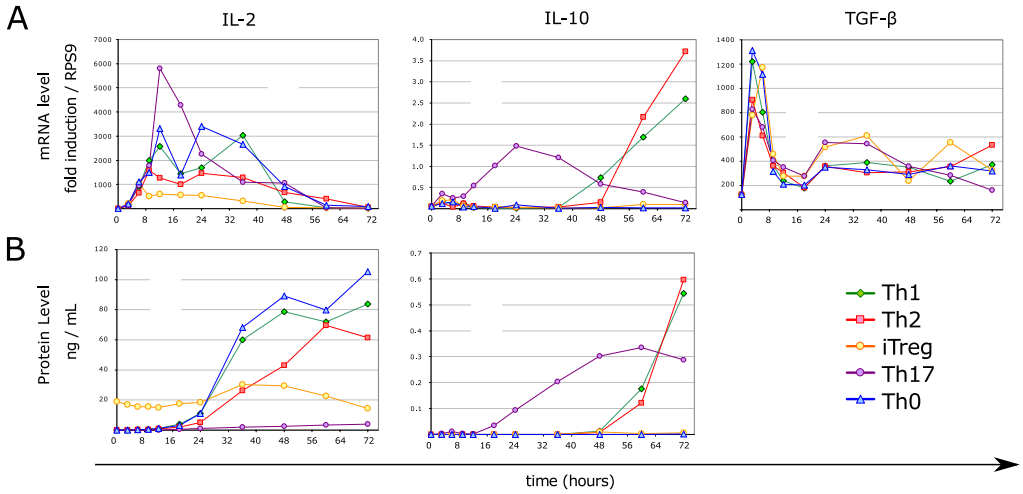
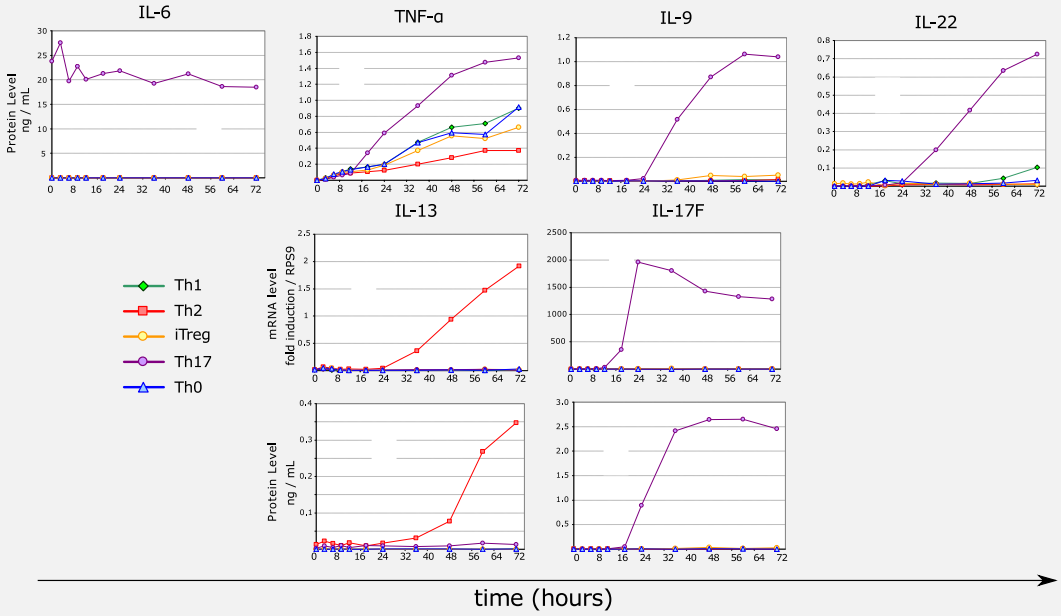


Figure 3.3: **Kinetics of IL-2, IL-10 and TGF-β expression during the first 3 days of differentiation**, in Th0, Th1, Th2, iTreg and Th17 conditions. A- Kinetics or mRNA expression, followed by RT-qPCR and normalized to RPS9 gene expression. B- Cytokine level in the culture supernatant, measured by multiplex. Note: IL-2 was added to the iTreg condition at the beginning. Due to the presence of large amounts of latent TGF-β in the culture coming from the serum, secreted TGF-β in the supernatant could not be directly measured. 2 independent experiments.

Box S5 : Dynamics of additional cytokines.



Dynamics of additional cytokines, as mRNA level measured by qPCR (IL-13, IL-17-F), and as concentration in the supernatant measured by multiplex. 2 independent experiments.

Building a dynamical network Designing a mathematical model starts by a choice of hypotheses on how to represent a biological system into abstract quantities and equations. Different researchers would probably take different sets of hypotheses and scales of representation. Including all factors involved in the molecular pathways between the input cytokines to the final decision would require to include thousands of molecules, which are probably all important in a specific context, but can not be simulated.

Several studies have tried to reconstruct the T helper differentiation network by use of transcriptomic time-resolved kinetics as well as perturbations from siRNA or deficient backgrounds [109, 169], at a genome-wide scale and without bias. These approaches unfortunately strongly overestimated the amount of interactions, because they rely on the time profile of factors to find interactions: an early gene can activate a later gene but a later gene can not activate an early gene, and this is not enough to reveal real, functional interactions.

In our study, some observations made this approach not suitable: 1/ different factors can have extreme levels of expression, with some factors being active at very low, even undetectable levels such as IFN-γ, while some other factors need to reach high levels to be effective (ex: Rorγt); 2/ the presence of delays, that create time uncorrelation between factors which actually interact, as we observed in the case of Foxp3 or cytokine secretion. This requires special care in term of modeling [231], and 3/ unless measuring at the same time mRNA and proteins, the presence of auto-activations such as Gata3 activating its own transcription can hardly be detected by such methods.

Interestingly, as an alternative approach, an automated method was used in [174] to compare the kinetics of a transcriptome following Th17 differentiation with interactome data from databases, in order to reconstruct sub-networks in accordance with the data, and using a ranking strategy to simulate only the key players, which succeeded into a quite reduced network. Still, these techniques try to represent every single mechanistic interaction steps, and it is very likely that the neglected factors, or interactions that remain to be discovered would change the properties of the regulatory networks once updated.

To avoid this problem as much as possible, we decided to represent only the key factors already described to control T helper differentiation, and the cytokines either added or produced as feedback. Instead of representing the exact mechanisms linking them, we use abstract activations or inhibitions when there is one or more, direct or indirect pathways (provided they don't cross another simulated factor). Therefore, each arrow represent the empirical effect that a factor has on another one, but we don't exactly specify by which mechanism. We followed a minimalist strategy, starting by published interactions, and by

including only required empirical interactions to explain the experimental data (for instance, linking the transient TCR peak to several genes).

In order to extract the dynamical properties of T helper cell differentiation from our data, we developed a mathematical model based on literature restricted to the mRNA and protein levels of the main transcription factors (T-bet, Gata3, Rorγt, Foxp3), and the main cytokines that have a feedback effect (IL-4, IFN-γ, IL-21, IL-2 and TGF-β). The inducing cytokines (IL-12, IL-6 and IL-1b) were also included in the simulations as they are added to the culture, but are supposedly not produced during differentiation. The most important pathways controlling differentiation (Explained in the introduction) are incorporated in the network (Figure 3.4) and were converted into Ordinary Differential Equations (ODEs) to simulate the kinetics of each variable (Box S9).

We could find published mechanisms allowing each transcription factor to inhibit every other one, therefore we draw inhibitions for all of them. When there were multiple mechanisms doing an activation or inhibition, they were pooled in one arrow. The model then captures the combined kinetics of both of them, without knowing which one is active.

Additional interactions were required from the data, and where taken as additional hypotheses:

- H1: As the mRNA of many genes showed a transient peak in all conditions due to TCR stimulation, we included a 'TCR transient signal' as a peak, and added a linear transcriptional activation to all target genes. All the genes of the network require TCR to be expressed, but these particular genes have a transient effect on top.

- H2: The decrease of Gata3 in the Th1 condition compared to Th0/iTreg couldn't be due to T-bet directly, because T-bet followed the same dynamics during Th1 and Th0/iTreg differentiations during the two first days (Figure 3.1). Therefore, the inhibition of Gata3 is specifically present in Th1 condition but not Th0. We propose it to be due (directly or indirectly) to IL-12, and therefore we add an inhibition from IL-12 to Gata3.

- H3: It has been shown that Gata3 is ubiquitinylated downstream TCR and would explain why its mRNA levels dropped. We therefore included a linear effect of TCR increasing Gata3 degradation.

Finally, we realized that from this network, several mechanisms were not active. First, in iTreg versus Th0 condition, the kinetics of T-bet and Gata3 were exactly the same, suggesting the inhibition of Foxp3 to T-bet and Gata3 were not active. It is consistent with the fact that

iTregs expressed low levels of T-bet and Gata3. However, such mechanisms could depend on additional stimuli and could have an importance in other conditions.

In order to explain the second peak of T-bet, IL-2 was a good candidate as an activator. STAT5 has been shown to bind to the T-bet promoter and to be supporting Th1 differentiation [232]. The model could fit T-bet profiles thanks to adding an activation from IL-2 to T-bet (not shown), showing it is a plausible explanation, though other ones could also be. However, blocking (Box S4) or adding IL-2 had no impact (data not shown) on T-bet expression at mRNA and protein level, so we discarded this activation from the network. Strikingky, even if IFN-γ was not detected early in the supernatant, blocking IFN-γ from the beginning or at different time-points (Box S4) abrogated T-bet upregulation after 24 hours, showing that, even if it is not detected, IFN-γ is the activator responsible for late T-bet upregulation, consistently with previous studies [111, 217], and therefore, the network didn't require any modification regarding the Th1 positive feedback loops.

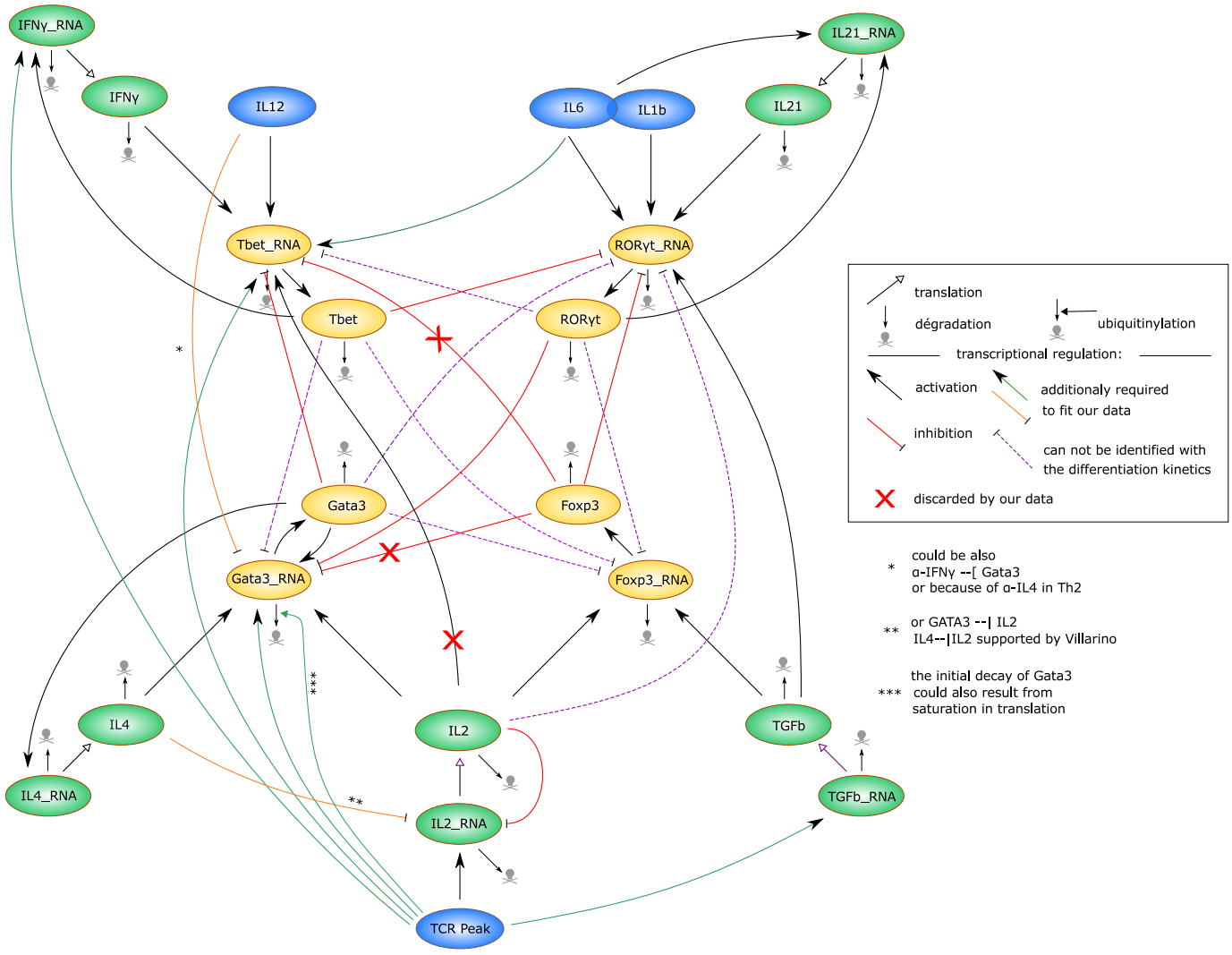


Figure 3.4: **Network of interactions between the critical players of T helper differentiation.** The variables (mRNA, intracellular protein for transcription factors and secreted protein for cytokines) are shown as nodes of the network. The biological processes targeting them are represented as arrows: degradation, translation, transcription, regulation of transcription (activation or inhibition). The regulation of differentiation is mainly done through the activation and inhibition of transcription. Black (activation) and red and purple (inhibition) arrows are directly taken from literature (indirect or indirect) allowing a gene to regulate another one.

Fitting and quantification of delays The regulatory network comes with many unknown parameters: degradation rates, translation rates, activation thresholds, etc. and a parameter estimation phase has to be performed to find realistic parameter values from the data.

As a first approach, a parameter estimation step was performed with all the parameters, but failed to find a realistic set of parameters (i.e. for which the simulation are similar enough to the differentiation kinetics), likely due to the high dimensionality of the problem (not shown).

Measuring both mRNA and protein levels from the same experiment has the important benefit of uncoupling feedbacks: in the network, there is no direct feedback from the mRNA levels to themselves, or from a protein levels to themselves. Therefore, the network should be able to simulate the mRNA kinetics from the protein data and to simulate the protein levels from the mRNA data. We then follow the iterative fitting procedure proposed in [214] (Box S6), where the network is cut into small sub-networks: each variable is fitted separately from the linear interpolation of the other variables from data (local fit), which allows to restrict the range of parameter values impacting this variable. Then, the whole network can be fitted (global fit) by constraining the parameter values to be in the range of the local fittings.

From the experimental, and thanks to this method, several temporal uncoupling could be observed between mRNA and protein levels, especially for T-bet, Gata3, Rorγt, IL2 and IL-21 (Figure 3.1 and 3.2). We first wondered if they could arise from the linear rates in the model (See equations in Box S9).

Local fittings of IL-2 protein profiles from IL-2 mRNA kinetics couldn't reproduce the experimental curve, as shown in (Figure 3.5). It means that the production of IL-2 in the supernatant from the mRNA (including translation and secretion), can not be explained by a linear (constant) 'translation and secretion' combined rate.

Similarly, in the case of the transcription factors, by the use of the local fittings, even if the mRNA was present at early time-points, the protein profile could not be explained by a constant translation rate (data not shown), and it therefore means that the translation rate is much lower in early time-points than observed at later time-points.

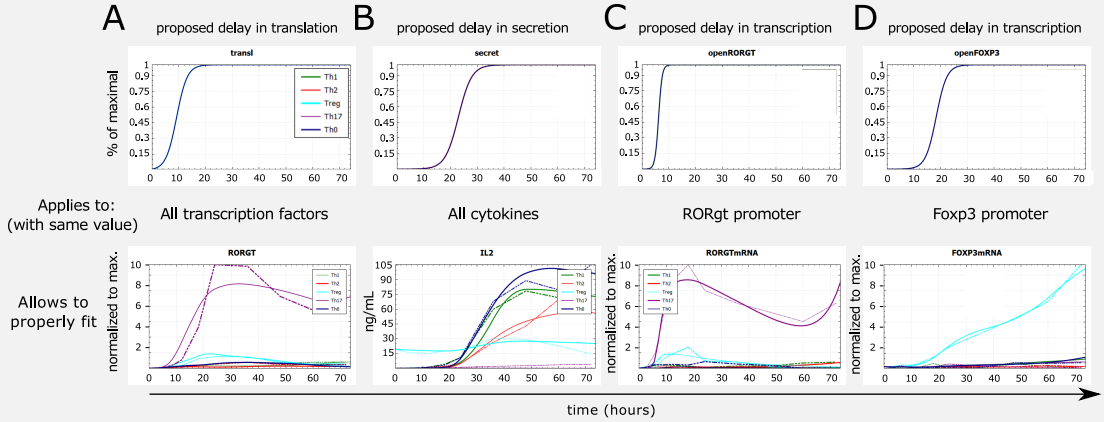
Delays in transcription could also be observed in the case of IL-2, IL-21, Rorγt and Foxp3. Indeed, for all of them, all the necessary signals for their production were present from the very beginning, and the model would always simulate a fast growing kinetics that can not fit the experimental kinetics that require more time (not shown). In the case of Foxp3 and Rorγt transcription, it was in agreement with the cells being yes/no producers in early time-

points (Box S3), and suggests the role of epigenetic control of their locus such as methylation [64, 233]. The delay downstream TCR to transcribe IL-2 could also arise from the time to demethylate its promoter [234] or might be due to other activators or inhibitors of IL-2 downstream TCR, which might need time to become effective or repressed, respectively.

In both cases, these latencies needed to be incorporated into the model, and we decided, as a simplest way, to give a translation capacity and a combined 'translation and secretion' capacity over time, parameterized as a sigmoid function with the critical time where the process gets activated. The different capacities profiles are listed in (Box S6). These sigmoidal functions therefore incorporate the time dynamics of most complex mechanisms without explicitely naming nor modeling such mechanisms.

To be fair, we applied the same secretion delay (identified for IL-2) to all the cytokines, and it was consistent with the dynamics of other cytokines (especially IL-21 levels in the supernatant; not shown). Therefore, the translational capacity curve was taken to be identical for all mRNAs to transcription factors, and the 'secretion' capacity was taken to be the same for all cytokines.

Box S6 : Delays implemented in the model, and local fitting quality



A. The time-dependent translation capacity applies to all transcription factors, and allows to fit properly Rorγt, T-bet and Gata3 protein dynamics. Rorγt is shown as example. B. The combined 'translation and secretion' capacity applies to all cytokines, and allows to simulate the kinetics of the early cytokines (IL-2 and IL-21). IL-2 is shown as example, as in Figure C5. C. and D. Transcriptional capacity of rorc, foxp3, IL-2 and IL-21 promoters allow to reproduce the mRNA profiles (for IL-2 and IL-21, the effect is minor, and is not shown) . Note: The mRNA profiles and the transcription factor protein levels are normalized separately to their maximum value (from all conditions) to span in a range of [0 .. 10], in order to have a similar contribution in fittings, and because they don't technically represent absolute values. Cytokines protein levels are not normalized.

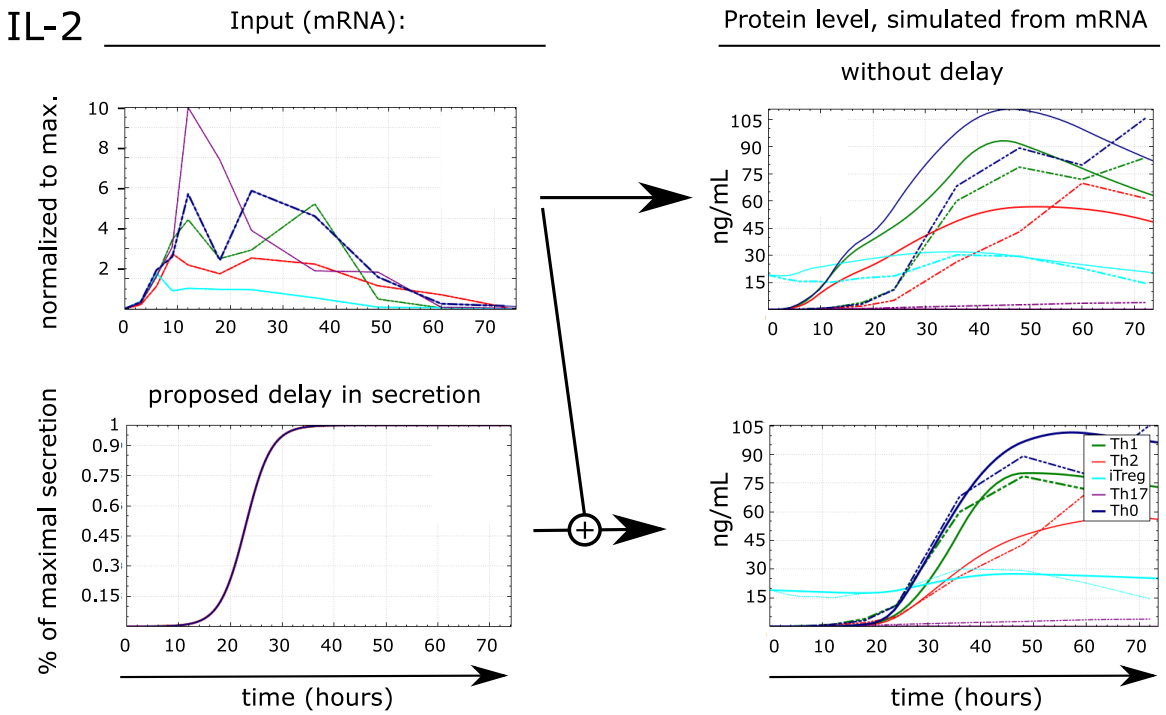
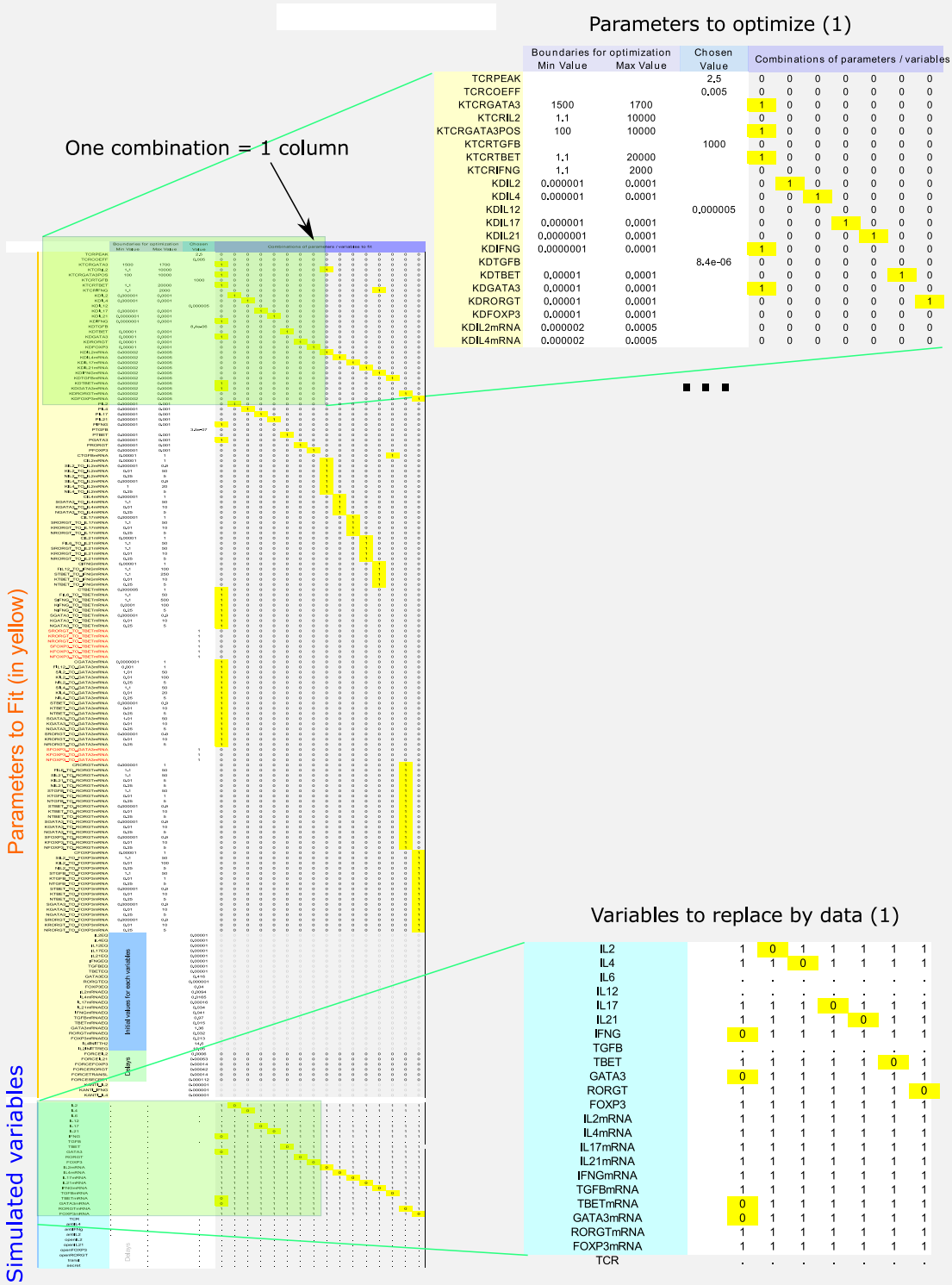


Figure 3.5: **quantifying the delay in IL-2 production.** Top: The experimental mRNA profile of IL-2 in different conditions (top left) together with the simulated best local fit for IL-2 cytokine levels (top right, smooth curves) without explicit delay, as compared to experimental data (broken lines). Bottom left: best secretion capacity curve, and right: best fit, by including the secretion capacity in the model. The mRNA profiles and the transcription factor protein levels are normalized separately to their maximum value (from all conditions) to span in a range of $[0 \,..\, 10]$, in order to have a similar contribution in fittings, and because the measurements do not technically represent absolute values. Cytokines protein levels are not normalized.

Box S7 : Separate fitting strategy.



It is possible to 'isolate' a variable by replacing the curves of all the other variables by the interpolated experimental data. In this case, only the parameters impacting this variable have an impact on the simulations, and it can be fitted separately. For fitting the CD4 differentiation network, all the 'combinations' (columns) used for separate fitting are shown. For a combination, the parameters fitted are highlighted on the upper part, together with the variables that were overridden (replaced) by experimental data. For instance, in the second combination, only IL-2 was simulated while all other variables were replaced by experimental data, and the corresponding parameters were fitted: P_{IL-2} and KD_{IL-2}. The first combination includes both IFN-γ, Gata3 mRNA and T-bet, because undetected IFN-γ could have an impact on T-bet

(Box S3). Otherwise, interpolating data for IFN-γ would increase the noise in the early time-points, and it is better to replace it by simulated values. The parameters in red represent interactions that were discarded from the data, therefore they are put to a coefficient of 1 (no effect) for the fitting of canonical differentiations.

The model accurately reproduces the data By now including the aforementioned delays, the mathematical model was able to reproduce the kinetics of the main transcription factors (Figure 3.6) and cytokines (Box S8), as mRNA and protein. It shows that the hypotheses included the model are consistent with the data. The best parameter values are given in (Appendix Figure 7.4). An identifiability analysis was performed on all the parameters (Appendix, Figure 7.3 and 7.4) to see if the parameter values are fully constrained by the data, or if other values could still be consistent with the data. It revealed that most of the parameters regarding protein translation and degradation could be identified. However, in case of translational regulation, due to the complexity of the regulation of the promoters, the parameters of the hill functions were not identifiable. Having a robust estimation of such parameters would require to measure the transcription rate in the context of different concentrations of each of the activators / inhibitors, and was not performed here.

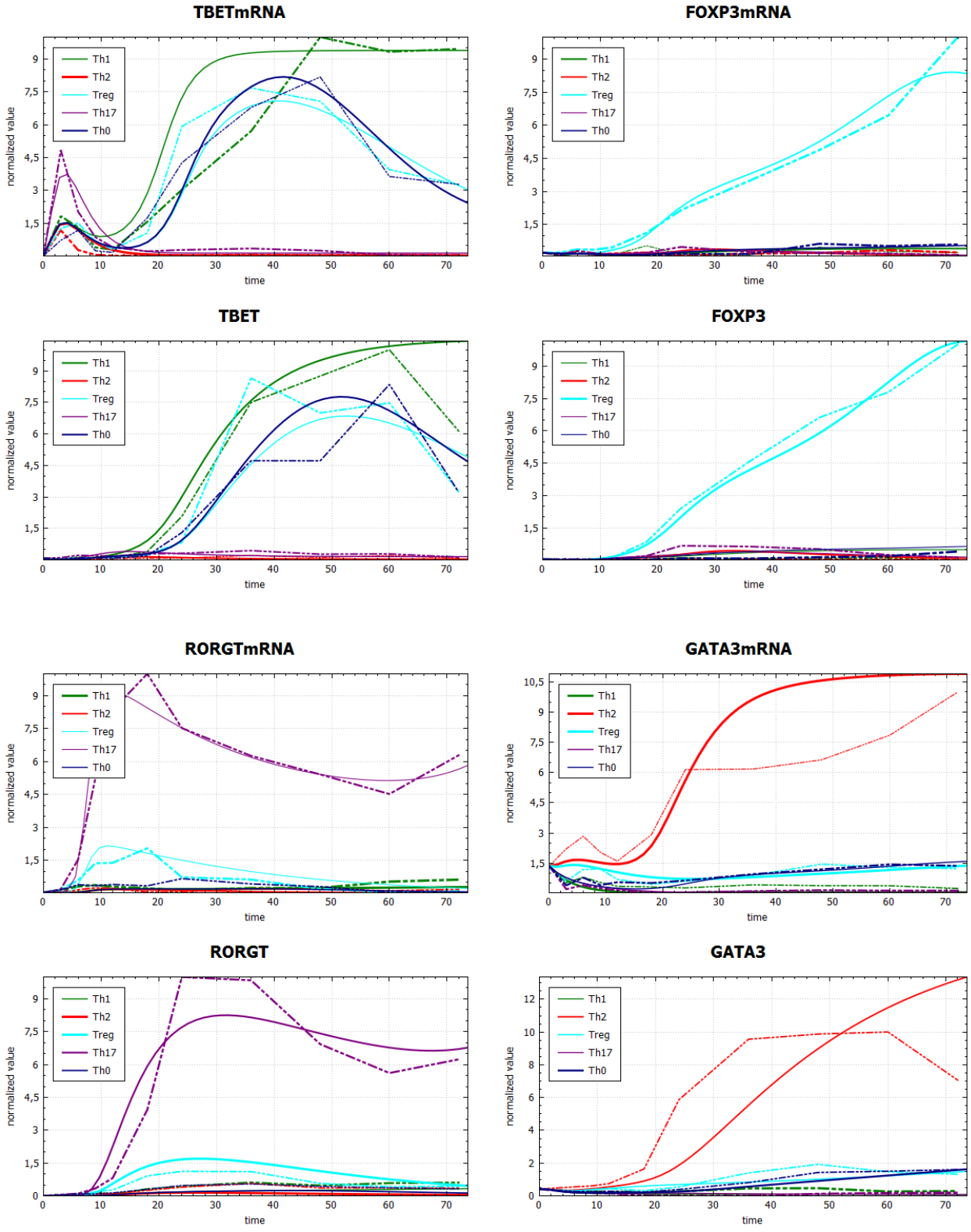
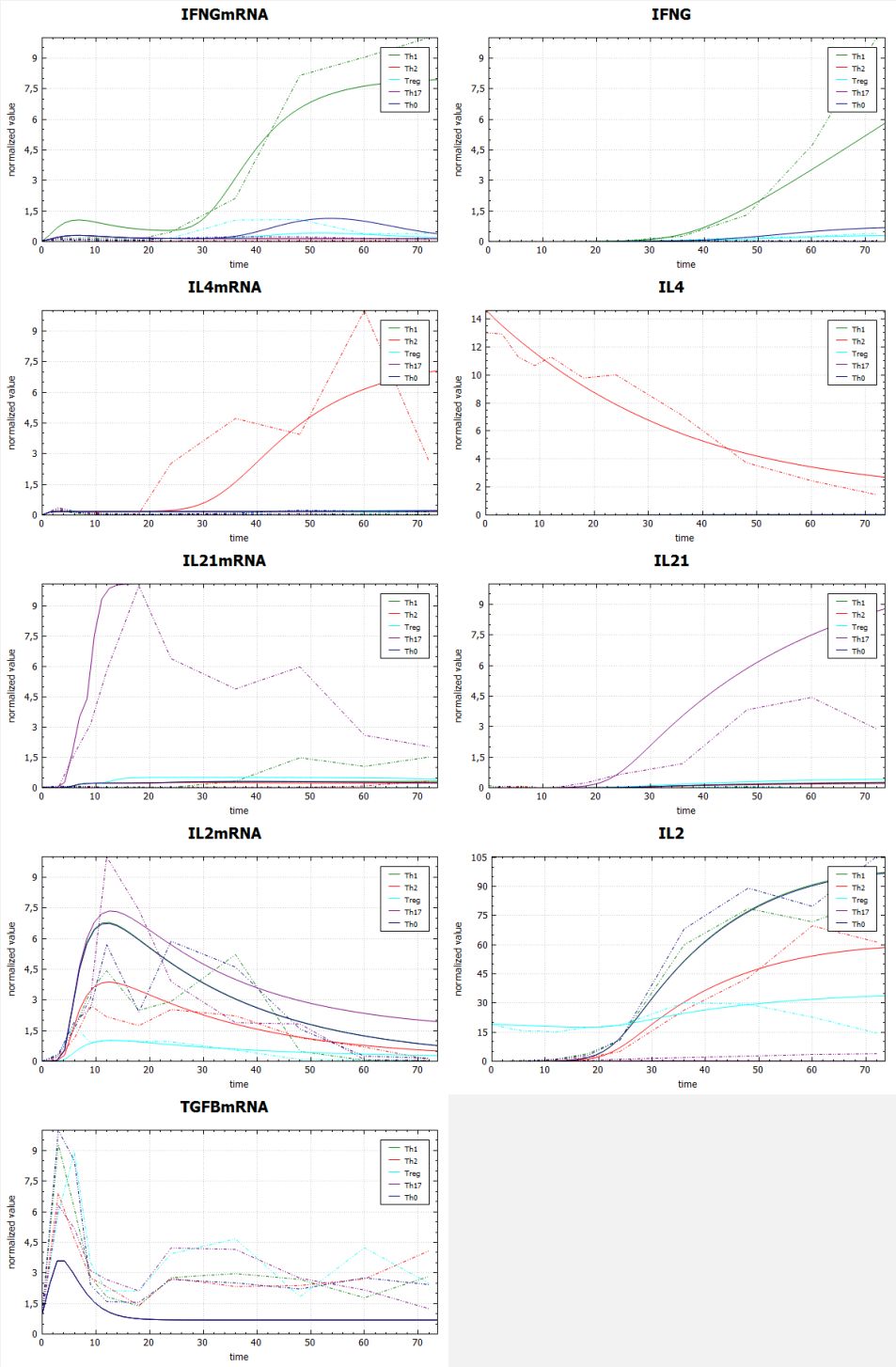


Figure 3.6: **Simulations from the best total fitting.** For each transcription factor, the simulations (smooth curves) are compared to the data (hard curves) in the 5 conditions of differentiation. The experimental data is the one showed in (Figures 3.2 and 3.1). The experiments were performed two times, and the two replicates can be compared in (Appendix, Figure 7.1). Each variable for which the measurement was not absolute (mRNA, MFI), were normalized between [0..10] to have a balanced effect of each variable during the fitting.

Box S8 : Simulation of cytokine dynamics from the best fitted set of parameters.



Box S9 : Formulation of the model and list of equations

In this study, Ordinary Differential Equations were used to simulate the regulatory network. Some processes such as translation and degradation are simulated as linear processes and succeeded to reproduce the data in the separate fitting strategy. Cytokine signal transduction is represented by a generic 3-parameter hill function. Finally, the behavior of gene promoters in the presence of activators and inhibitors at the same time is globally unknown. We had to choose a simple, though general representation, and decided to model transcription as a product of separate hill functions for each activator or inhibitor. Each hill function starts at 1 when the activator (or inhibitor) is absent, allowing one activator to start transcription alone even if the other ones are absent. Multiplying the contributions of each transcription factor allows to have synergy between factors when multiple of them are present. We believe it is a general way to represent most possible behaviors of a gene.
Here, the equations with simplified notations are shown. For details, the full version is given in the Appendix. In (Figure 7.4), the complete and simplified parameter names are given together with the best values found by parameter estimation.

List of simulated variables

- the concentration of cytokines in the medium in ng/mL : $[IL2]$, $[IL4]$, $[IL6]$, $[IL12]$, $[IL17]$, $[IL21]$, $[IFNG]$, $[TGFB]$;
- the concentration of transcription factors inside the cell : $[TBET]$, $[GATA3]$, $[RORGT]$, $[FOXP3]$. There is no unit as it follows the scale of fluorescence intensity measured by FACS;
- mRNA level inside the cell, in fold induction compared to RPS9 mRNAs at the same time-point : $[mIL2]$, $[mIL4]$, $[mIL17]$, $[mIL21]$, $[mIFN]$, $[mTGFB]$, $[mTBET]$, $[mGATA3]$, $[mRORGT]$, $[mFOXP3]$;
- the concentration of free blocking antibodies in the medium in mg/mL : $[\alpha IL4]$, $[\alpha IFNg]$, $[\alpha IL2]$;

List of time-dependent mechanisms with pre-defined functions

- the peak of signaling following TCR triggering : $TCR(t)$,
- the state of processes showing saturation or delay. All start very small (0.001) at t 0, and increase to 1 (full capacity) with time : $O_2(t)$, $O_{21}(t)$, $O_{fp3}(t)$, $O_{ror}(t)$, for the chromatin opening states of these genes, $T(t)$ the translational capacity, and $S(t)$ the secretion capacity.

Equations

1. Cytokine levels in the medium are impacted by :
    - production through translation (P), limited by the secretion capacity of the cell changing with time ($S(t)$),
    - degradation (D)
    - binding to blocking antibodies when present in the culture (B)

$$\frac{d[IL2]}{dt} \quad -D_2.[IL2]\,P_2.S(t).[mIL2]-B_{\alpha IL2}.[IL2].[\alpha IL2] \tag{3.1}$$
$$\frac{d[IL4]}{dt} \quad -D_4.[IL4]P_4.S(t).[mIL4]-B_{\alpha IL4}.[IL4].[\alpha IL4] \tag{3.2}$$
$$\frac{d[IL6]}{dt} \quad 0 \tag{3.3}$$
$$\frac{d[IL12]}{dt} \quad -D_{12}.[IL12] \tag{3.4}$$
$$\frac{d[IL17]}{dt} \quad -D_{17}.[IL17]\,P_{17}.S(t).[mIL17] \tag{3.5}$$
$$\frac{d[IL21]}{dt} \quad -D_{21}.[IL21]\,P_{21}.S(t).[mIL21] \tag{3.6}$$
$$\frac{d[IFN\gamma]}{dt} \quad -D_{\gamma}.[IFN\gamma]\,P_{\gamma}.S(t).[mIFN\gamma]-B_{\alpha IFN\gamma}.[IFN\gamma].[\alpha IFN\gamma] \tag{3.7}$$
$$\frac{d[TGF\beta]}{dt} \quad -D_{\beta}.[TGF\beta]\,P_{\beta}.S(t).[mTGF\beta] \tag{3.8}$$
$$\tag{3.9}$$

$IL6$ and $IL12$ are not produced by T cells during in vitro-differentiation (at least, not detected). Commercial $IL6$ does not show any degradation in my cultures.

2. Transcription factor protein levels in the cells are impacted by :

   - degradation D, increased in the case of ubiquitination (K_{Ub}),
   - production from translation (P), limited by the translational capacity of the cell ($T(t)$) which is saturated in the first hours

$$\frac{d[TBET]}{dt} \quad -D_T.[TBET]\,P_T.T(t).[mTBET] \tag{3.10}$$
$$\frac{d[GATA3]}{dt} \quad -D_G.[GATA3].(1\,K_{Ub}.[TCR])\,P_G.T(t).[mGATA3] \tag{3.11}$$
$$\frac{d[RORGT]}{dt} \quad -D_R.[RORGT]\,P_R.T(t).[mRORGT] \tag{3.12}$$
$$\frac{d[FOXP3]}{dt} \quad -D_F.[FOXP3]\,P_F.T(t).[mFOXP3] \tag{3.13}$$
$$\tag{3.14}$$

3. mRNA levels in the cells are impacted by

   - degradation Dm,
   - production from transcription, with basal coefficient (C), regulated by activators or inhibitors (with linear effect K, or using hill functions with parameters K:threshold, N:slope and S:max fold induction), and limited by the chromatin opening of specific locus ($O_X(t)$)

   Each activation from a protein $[A]$ is represented by a Hill function :

$$Activ([A],S,K,N)\ (1\ (1-S).\frac{[A]^N}{K^N\ [A]^N})$$

   Similarly, an inhibition from a factor $[I]$ is represented by a Hill function :

$$Inhib([B],S,K,N)\ (S\ (1-S).\frac{K^N}{K^N\ [I]^N})$$

   For the cytokine that are not modelled over time, the hill function linking the cytokine concentration to the transcriptional activity is replaced by only one factor S, by using the function $\delta([A],S)\ S$ if $[A]\ 0]$ (effect) and 1 otherwise (no effect).

$$\frac{d[mIL2]}{dt} - D_{m2}.[mIL2]\, C_2.O_2(t).(1\, K_{TCR\rightarrow IL2}.[TCR]).\\ Inhib([IL2],S_1,K_1,N_1).Inhib([IL4],S_2,K_2,N_2) \tag{3.15}$$

$$\frac{d[mIL4]}{dt} - D_{m4}.[mIL4]\, C_4.\\ Activ([GATA3],S_3,K_3,N_3) \tag{3.16}$$

$$\frac{d[mIL17]}{dt} - D_{mIL17}.[mIL17]\, C_{17}.Activ([RORGt],S_3,K_3,N_3) \tag{3.17}$$

$$\frac{d[mIL21]}{dt} - D_{m21}.[mIL21]\, C_{21}.O_{21}(t).(\delta([IL6]\ 0,S_4)).\\ Activ([RORGt],S_5,K_5,N_5) \tag{3.18}$$

$$\frac{d[mIFN\gamma]}{dt} - D_{m\gamma}.[mIFN\gamma]\, C_\gamma.(\delta([IL12]\ 0,S_6)).\\ (1\, K_{TCR\rightarrow IFN\gamma}.[TCR]).Activ([TBET],S_7,K_7,N_7) \tag{3.19}$$

$$\frac{d[mTGF\beta]}{dt} - D_{m\beta}.[mTGF\beta]\, C_\beta.(1\, K_{TCR\rightarrow TGF\beta}.[TCR]) \tag{3.20}$$

$$\frac{d[mTBET]}{dt} - D_{mT}.[mTBET]\, C_T.(1\, K_{TCR\rightarrow TBET}.[TCR]).(\delta([IL6]\ 0,S_8)).\\ Activ([IFNG],S_9,K_9,N_9).Inhib([GATA],S_{10},K_{10},N_{10}).\\ Inhib([RORGT],S_{11},K_{11},N_{11}).Inhib([FOXP3],S_{12},K_{12},N_{12}) \tag{3.21}$$

$$\frac{d[mGATA3]}{dt} - D_{mG}.[mGATA3]\, C_G.(1\, K_{TCR\rightarrow GATA3,POS}.[TCR]).(\delta([IL12]\ 0,S_13)).\\ Activ([IL2],S_{14},K_{14},N_{14}).Activ([IL4],S_{15},K_{15},N_{15}).\\ Inhib([TBET],S_{16},K_{16},N_{16}).Activ([GATA],S_{17},K_{17},N_{17}).\\ Inhib([RORGT],S_{18},K_{18},N_{18}).Inhib([FOXP3],S_{19},K_{19},N_{19}) \tag{3.22}$$

$$\frac{d[mRORGT]}{dt} - D_{mR}.[mRORGT]\, C_R.O_{ror}(t).(\delta([IL16]\ 0,S20)).\\ Activ([IL21],S_{21},K_{21},N_{21}).Activ([TGFB],S_{22},K_{22},N_{22}).\\ Inhib([TBET],S_{23},K_{23},N_{23}).Inhib([GATA],S_{24},K_{24},N_{24}).\\ Inhib([FOXP3],S_{25},K_{25},N_{25}) \tag{3.23}$$

$$\frac{d[mFOXP3]}{dt} - D_{mF}.[mFOXP3]_{(t)}\, C_F.O_{fp3}(t).Activ([IL2],S_{26},K_{26},N_{26}).\\ Activ([TGFB],S_{27},K_{27},N_{27}).Inhib([TBET],S_{28},K_{28},N_{28}).\\ Inhib([GATA],S_{29},K_{29},N_{29}).Inhib([RORGT],S_{30},K_{30},N_{30}) \tag{3.24}$$

4. Remaining doses of free blocking antibodies The antibodies are stable (no degradation), and the annealing is fast (K_B is chosen big) and complete, meaning the complexes cytokine-antibody will stay bound. Since the antibodies are put in saturating concentrations, the cytokines are annealed very fast. But for later predictions, low antibody doses can be tried.

$$\frac{d[\alpha IL2]}{dt} \quad -B_{\alpha IL2}.[IL2].[\alpha IL2] \tag{3.25}$$
$$\frac{d[\alpha IL4]}{dt} \quad -B_{\alpha IL4}.[IL4].[\alpha IL4] \tag{3.26}$$
$$\frac{d[\alpha IFN\gamma]}{dt} \quad -B_{\alpha IFN\gamma}.[IFN\gamma].[\alpha IFN\gamma] \tag{3.27}$$
$$\tag{3.28}$$

5. Time-dependent mechanisms (with pre-defined kinetics)

   - TCR peak, with the peak at time $TCRPEAK$ and a maximum of $TCRCOEFF$

$$[TCR]_{(t)} \quad (TCRCOEFF * (t/3600)) * exp^{-\frac{\lambda.t}{3600}}$$
$$\lambda \quad \frac{1}{TCRPEAK}; \tag{3.29}$$

   - For the processes showing a time-dependence (translation, secretion and chromatin opening), a sigmoid function is used, whose kinetics is determined by the $FORCE$ parameters.

$$\frac{d[S(t)]}{dt} \quad F_s.S(t).(1-S(t)) \tag{3.30}$$
$$\frac{d[T(t)]}{dt} \quad F_t.T(t).(1-T(t)) \tag{3.31}$$
$$\frac{d[O_2(t)]}{dt} \quad F_2.O_2(t).(1-O_2(t)) \tag{3.32}$$
$$\frac{d[O_21(t)]}{dt} \quad F_{21}.O_{21}(t).(1-O_{21}(t)) \tag{3.33}$$
$$\frac{d[O_{ror}(t)]}{dt} \quad F_{ror}.O_{ror}(t).(1-O_{ror}(t)) \tag{3.34}$$
$$\frac{d[O_{fp3}(t)]}{dt} \quad F_{fp3}.O_{fp3}(t).(1-O_{fp3}(t)) \tag{3.35}$$
$$\tag{3.36}$$

Prediction: IL-2 controls the T-bet-Gata3 balance in Th0 and iTreg cells As a first set of predictions, the model was used to simulate the effect of performing the differentiations conditions, together with a specific dose of IL-2 from the beginning of the culture (Figure 3.7). In the Th0 condition, counter-intuitively, a high amount of IL-2 induced a reduced final amount of IL-2, due to its auto-inhibition. This was true for all subsets (not shown). The model predicted no effect of IL-2 on Th1 condition (not shown). In the Th2 condition, the dynamics of Gata3 and IL-4 expression in silico were faster with higher doses of IL-2 but didn't impact the later differentiation. Finally, the dose of IL-2 impacted the balance between T-bet and Gata3 in the iTreg condition, with higher doses inhibiting T-bet, indirectly due to Gata3 upregulation. It suggests that, paradoxically, for dampening IL-2 production by T cells, an initial low dose of IL-2 is beneficial. It has been proposed to use low dose IL-2 therapies to selectively boost Tregs but not Tconvs based on their different sensitivity to IL-2 [235]. One could speculate that such therapies also reduce Tconv expansion by blocking their own IL-2 production, independently of Tregs.

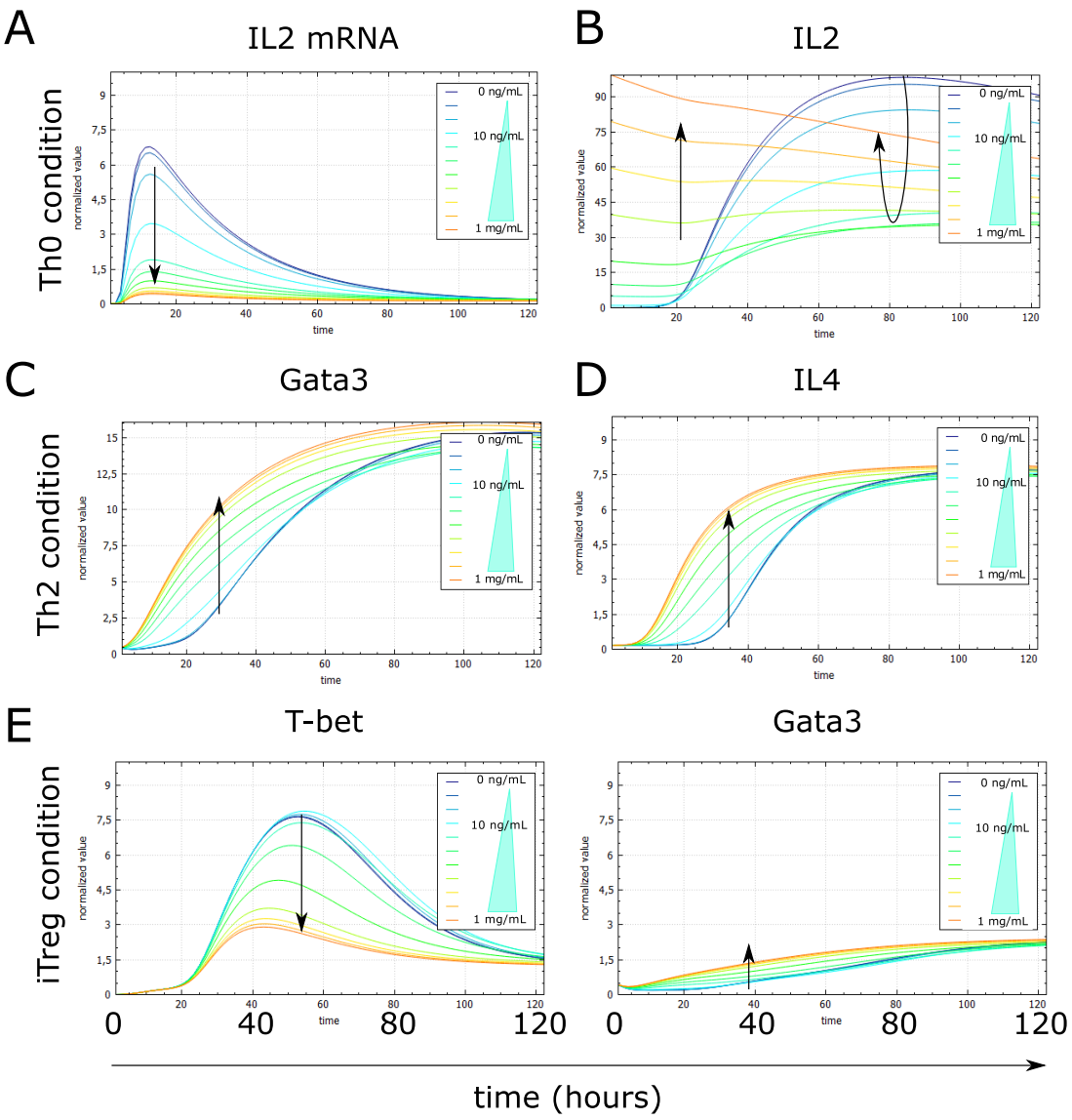


Figure 3.7: **: Effect of performing differentiation cultures with specific doses of IL-2 in silico.** A-D Simulations for the kinetics of transcription factors at the mRNA and protein levels are depicted under different conditions. Note: the Th17 condition contains anti-IL-2 and is not simulated here. E Simulations of IL-2 kinetics at mRNA and protein level due to the presence of different doses of IL-2. Curves are the same for Th1, iTreg and Th0 conditions.

Prediction: the consumption of IL-2 by bystander cells impacts the T-bet/Gata3 balance in iTregs It has been proposed that the consumption of IL-2 by regulatory T cells can suppress the activation of T helper subsets [201]. We wondered whether this mechanism has an impact on the balance between transcription factors inside T cells. It was modelled by increasing the degradation rate of IL-2 in the medium, and led to the kinetics of transcription factors shown in (Figure 3.8). While IL-2 consumption had no effect on Th1 and Th17 subsets (because of the presence of anti-IL-2 for the latter), the model predicted that an increase in IL-2 consumption would reduce Gata3 levels in Th2 and iTreg cells, while increasing the amount of T-bet in iTregs.

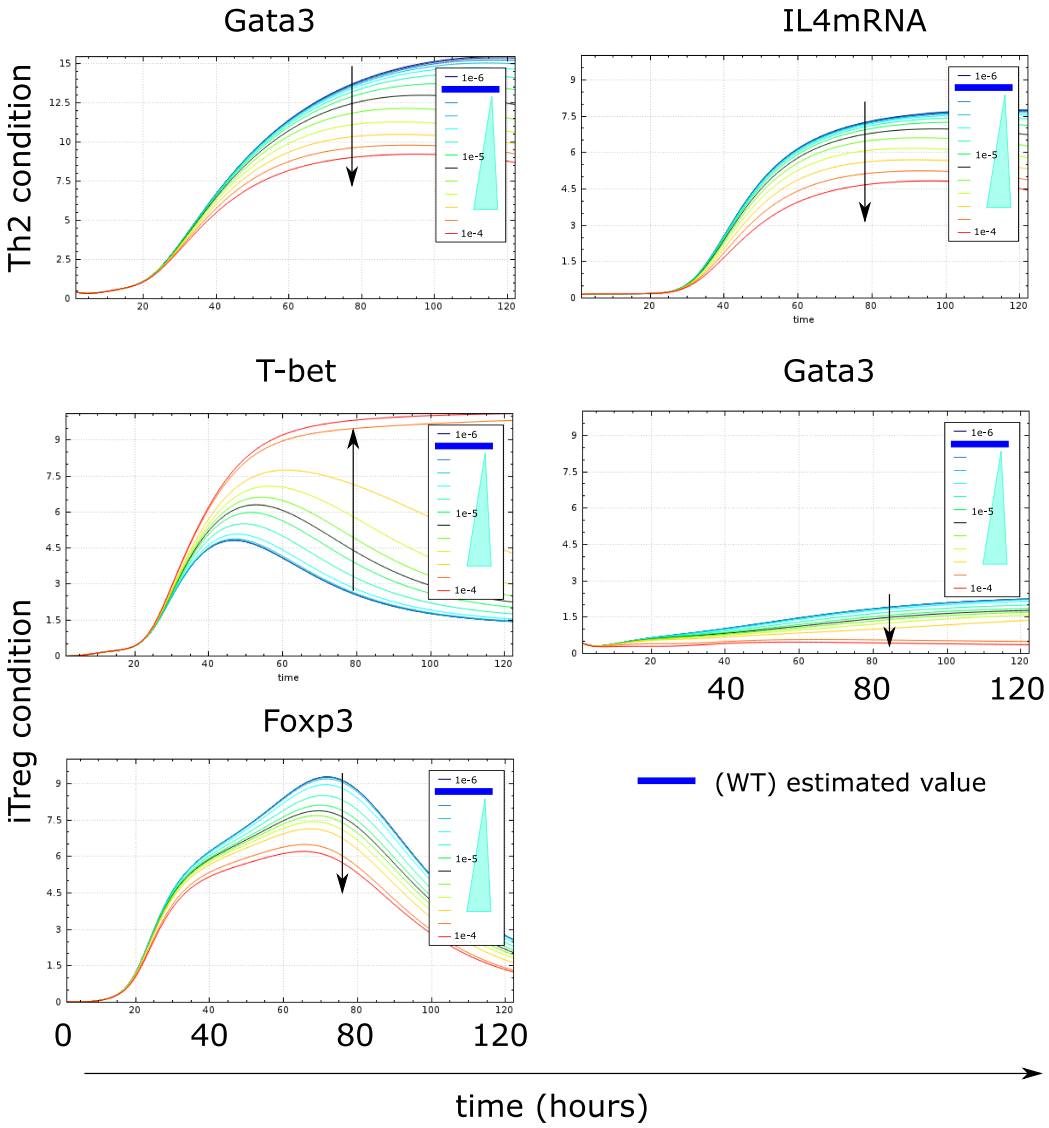


Figure 3.8: **Kinetics of transcription factors during differentiation into Th2 and iTreg conditions depending on the degradation rate of IL-2**, as a mimicry for IL-2 consumption during IL-2-mediated suppression.

CHAPTER 4

ASSESSING T HELPER PLASTICITY IN VITRO AND IN SILICO

4.1 Introduction

After training the mathematical model with the experimental dynamics of differentiation, we decided to test its robustness according to a new independent set of data.

An interesting property of T helper cells is their plasticity to redifferentiation under specific conditions, though this has not been assessed at the early time points for which the model was designed and validated.

We investigated the plasticity of cells in vitro, by changing the polarizing condition at 20 hours after the start of differentiation, just after the induction of master transcription factors, but before cell division. A very high plasticity could be observed. All the cells could acquire the expression of the new transcription factor independently of the previous inducing condition. Similarly, they could express IL-17 and IFN-γ upon switching into Th17 and Th1 condition respectively, except in the Th17 to Th1 switch, showing that early Th17 differentiation did not allow for further IFN-γ production. The same experimental settings were simulated in silico in parallel, to evaluate if the model can predict differentiation and plasticity in these new conditions without prior knowledge of the data. The model predicted a high plasticity to similar extend than the experimental data, but could not accurately fit the kinetics of transcription factors.

It suggests that some properties of the differentiation network could not be extracted only from the kinetics of the main factors during canonical differentiations. Instead, this switching dataset contains additional information that will be given to the mathematical model as an additional training dataset.

Finally, in order to design new experiments that could later validate the robustness of the network, and since 'old cells' are believed to be more stable in literature, the plasticity of T helper cells was assessed in a new, later settings: fully differentiated cells were co-cultured with other subsets, either without restimulation at day 3, or with restimulation, at day 10.

4.2 Results

Differentiating cells are plastic to repolarization even when the master transcription factors are highly expressed We wondered if differentiating cells would be plastic to repolarization into another subset. We chose a time-point of 20 hours to switch stimulation, such that the mRNA for the transcription factors is already induced, but the cells didn't divide yet. It avoids the effect of potential asymmetric divisions on the result [200], which are not included in the model and would be hard to interprete. Differentiation cultures into each subset were started for 20 hours, the supernatant was washed carefully and fresh medium was added with the cytokines of other conditions. The expression of the transcription factors was followed by flow cytometry after this perturbation (Figure 4.1). At 72 hours, the expression of cytokines was additionally assessed by cytokine staining for IFN-γ and IL-17 (Figure 4.2).

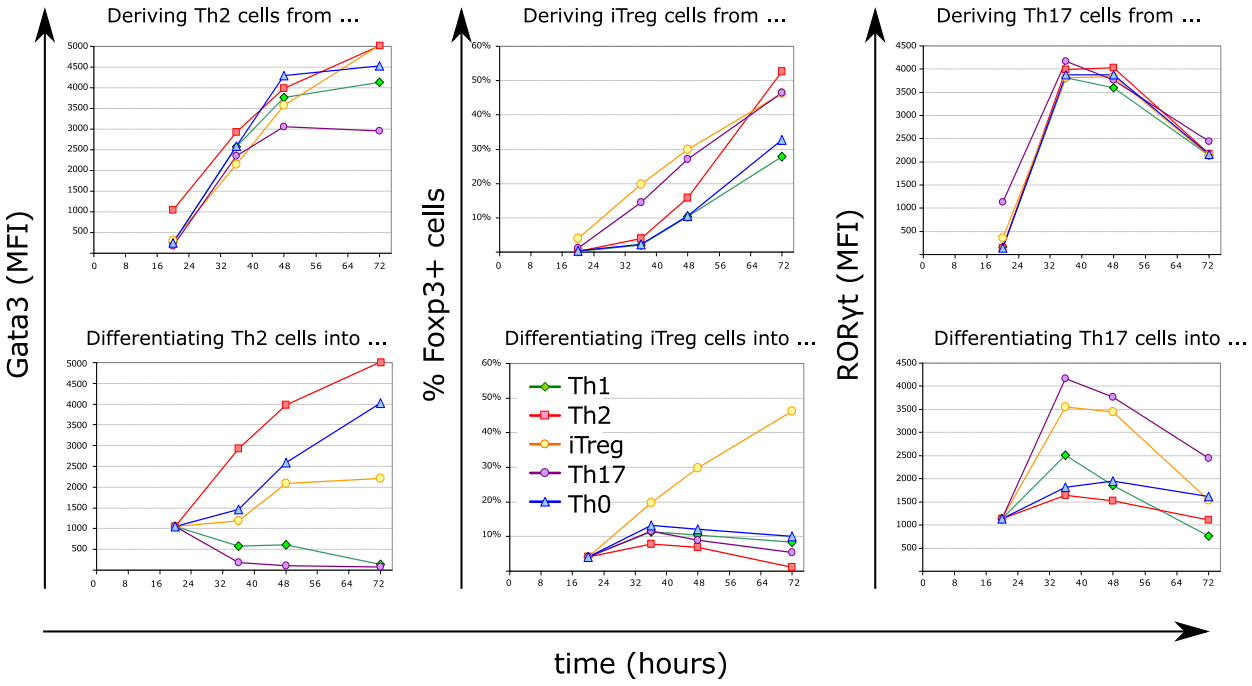


Figure 4.1: **Dynamics of transcription factors expression as protein levels after switching the differentiation condition at 20 hours**, measured by flow cytometry. 2 independent experiments. T-bet stainings showed poor quality (technical problems), and are not shown here.

At the transcription factor level, redifferentiation into Th2, Th17 and iTreg was possible independently of the previous inducing condition (Figure 4.1, upper panel), showing a full plasticity to acquire the new phenotype at 20 hours.

In contrast, the fate of the initially induced transcription factor depended on the subsequent condition (Figure 4.1, lower panel). Rorγt level was sustained to intermediate levels after an initial period of 20 hours of Th17 differentiation, independently of the later signals. Starting from an iTreg condition, low levels of Foxp3 were transiently maintained after switching to the other conditions but slowly decayed. Finally, in case of initial Th2 polarization, Th0 and iTreg conditions were permissive to sustained and increasing expression of Gata3 while Th1 and Th17 conditions fully inhibited its expression. It shows that, even if cells are plastic, they can keep a memory of their previous states in some of the conditions, and it is possible to generate double positive cells in vitro, such as iTregs with high expression of Gata3, or Gata3+ Rorγt+ cells at day 3. The stability of such double positive cells would need to be assessed.

At the cytokinic levels (Figure 4.2), Th1 redifferentiation lead to high amounts of IFN-γ production (Figure 4.2, upper panel), except after 20 hours of Th17 culture. When Th1 cells were challenged by other stimulations, IFN-γ expression was maintained to higher levels only in Th0 and Th1 condition but was suppressed by the iTreg, Th2 and Th17 inducing signals.

When the cells were redifferentiated into Th17 cells, a high level of IL-17+ producers was detected in all cases, consistant with the high expression of Rorγt in these conditions (Figure 4.2, lower panel). Interestingly, redifferentiating cells from an initial Th17 condition showed a reminiscence of IL-17 expression in iTreg and Th0 but not in Th1 or Th2 conditions (Figure 4.2, lower panel). This finding does not match with the Rorγt levels observed in (Figure 4.1), which are very similar between Th1, Th2 and Th0, revealing that, while Rorγt expression was dominant over other transcription factors, additional mechanisms decide for the expression of IL-17.

It has to be noted that the differentiation cultures contain blocking antibodies. In particular, in the Th1→Th17 and Th1→Th2 condition, anti-IFN-γ is included in the second condition, and might block IFN-γ production in an artificial way compared to an in vivo settings, devoid of blocking antibodies. Here, the experiment was designed to maximize the chance of plasticity (with blocking antibodies), but they would need to be reproduced without blocking antibodies to get more mechanistic insights.

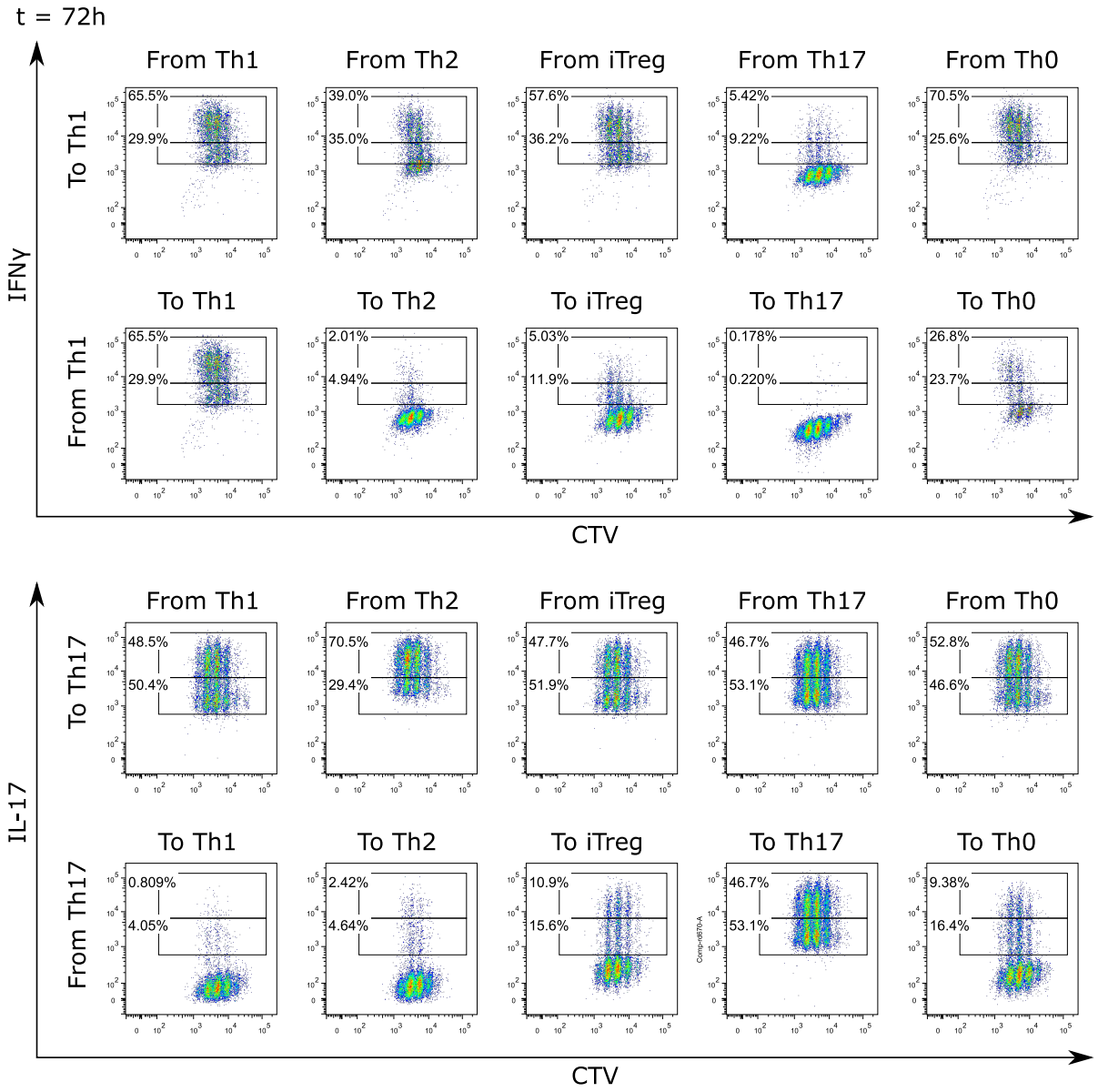


Figure 4.2: **Cytokine expression at day 3 by flow cytometry, when the culture condition was changed at 20 hours.** Proliferation is assessed by initial CTV labelling before initial stimulation, allowing a better separation of populations. Top: frequency of IFN-γ producers, separated into intermediate and high producers, as observed in [236]. Only the switching conditions from and to Th1 are shown. Bottom: frequency of IL-17 intermediate and high producers, similarly to the two populations observed for IFN-γ. Only the conditions switching from or to Th17 are shown. IL-17 was not observed in any other switching condition. 2 independent experiments.

The model qualitatively recapitulates early plasticity but fails to explain the curves. The mathematical model was used to predict the effect of condition switching at 20 hours. The simulated curves for transcription factor expression were compared with the experimental data (Figure 4.3). Note that the simulations were extended up to 5 days here.

Following T-bet expression over time, the model predicts that T-bet expression can be acquired upon switching to Th1 condition independently of the previous condition (Figure 4.3A). However, cells first simulated in the Th1 condition followed the same kinetics than T-bet in the new condition, as if nothing happened in the first 20 hours (see Figure 3.1 for comparison). Due to technical reasons, the experimental data is not available for T-bet over time upon switching. The predictions of the model are only partially consistent with the amount of IFN-γ producers (Figure 4.2, upper panel). Indeed, IFN-γ could be acquired after switching from most subsets but not Th17, while T-bet was predicted to be maintained in Th17 → Th1 condition. IFN-γ was kept only in Th1 and Th0 conditions but not iTreg when switching from Th1, while the model predicted similar T-bet profiles in the Th1→Th0 and Th1→iTreg condition. However, it doesn't mean that the model is wrong, and these inconsistencies between T-bet and IFN-γ might be true.

The predicted kinetics of Gata3 expression globally follow the experimental profiles (Figure 4.3B), where all cells could acquire Gata3 expression after switching to Th2 (though the model predicts a too slow upregulation in Th2 → Th17). The predicted switching from Th2 to Th1 and Th17 successfully showed Gata3 suppression. However, even if the model qualitatively predicts sustained levels of Gata3 in the Th2→Th0 and Th2→iTregs conditions, they are underappreciating the Gata3 amounts experimentally observed.

In the case of Foxp3 expression (Figure 4.3C), again, the model qualitatively predicted the accurate trends of switching, while it failed to predict that cells can express Foxp3 in the Th17 → iTreg condition. Interestingly, in all conditions, the model predicts that Foxp3 expression is not stable after three days.

The model also predicted a full plasticity towards acquiring Rorγt expression upon Th17 switching, but with too slow kinetics (Figure 4.3D). Finally, The model accurately reproduced the experimental kinetics of Rorγt when switching from Th17 to another subset. At later time-points (after day 3), it predicts that Rorγt will be stably expressed, except in Th17→iTreg condition. As a limitation, the model diverges in the Th17 (untouched) condition, which is not physiological.

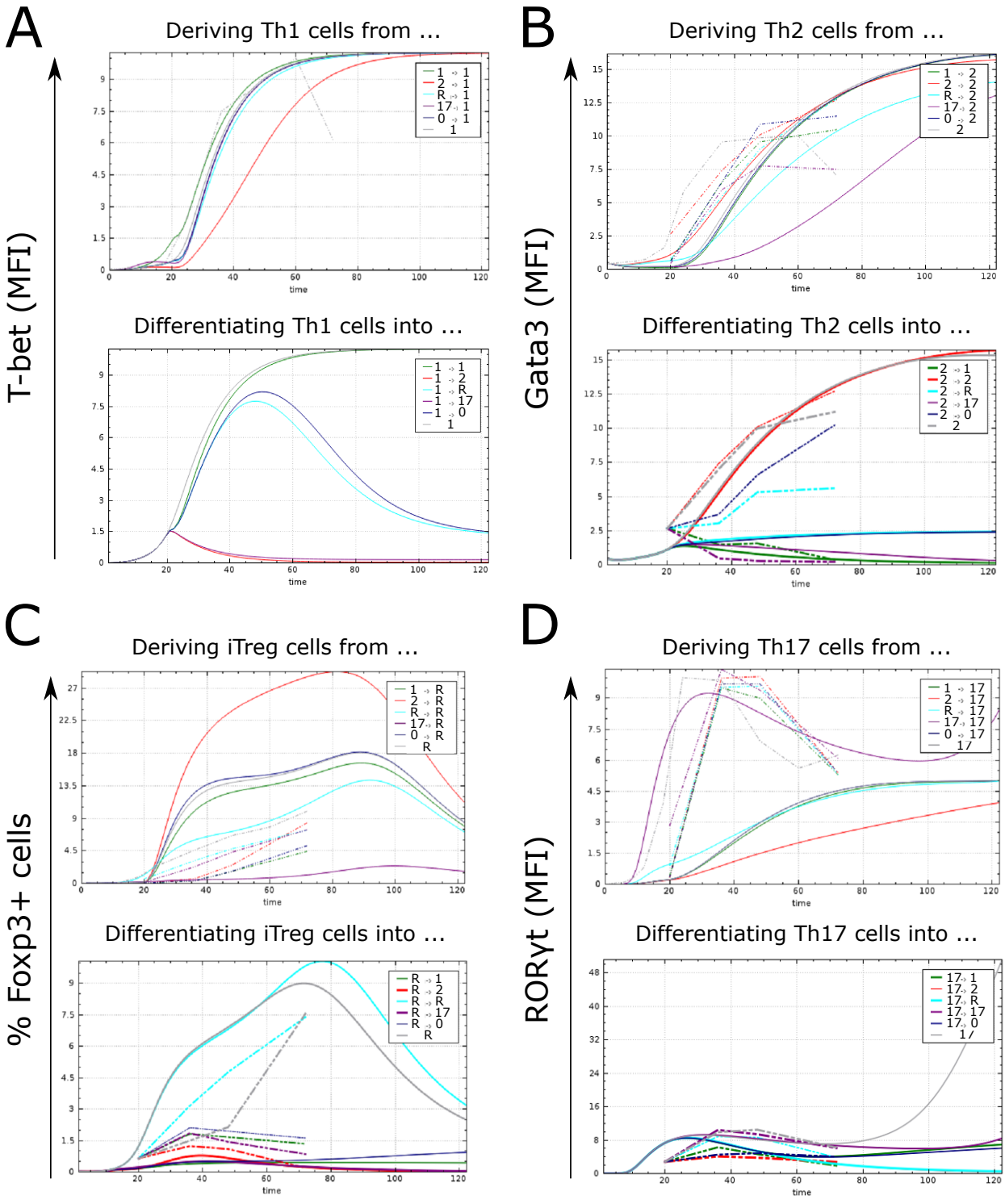


Figure 4.3: **Simulations for switching differentiation condition at 20 hours**, using the model and parameters obtained in chapter 3. The amount of transcription factor is displayed over time either simulated (smooth curves), or according to the experimental data of figure D1 (dashed curves). Each curve represent a different combination of an initial and later differentiation condition. An additional curve is shown (in gray), as a control where the cells are untouched (canonical differentiation, experimental data taken from chapter 3, Figure 3.1).

To conclude, the model, though being fitted from time-resolved experimental data of the canonical differentiations, was only partially able to recapitulate the early plasticity observed in this study. It shows that following the kinetics of elements of a network is not necessarily enough to fully determine this network, and that it is difficult to evaluate which data is required to completely determine a mathematical model. Indeed, only a minor number of parameters were identifiable from the data (Appendix, Figure 7.4). The kinetics of transcription factors upon early switching will be included in new fittings of the model, and an additional dataset will need to be generated to assess the predictive capacity of the model.

Three days of differentiation are sufficient for a stable phenotype in co-culture with other subsets. T helper cells initiate their differentiation in the lymphoid organs and then migrate in peripheral organs. We wondered what would happen if differentiated T helper cells from different subsets would encounter in periphery, and in particular if they would be stable, inhibit each-other, or induce a particular reprogramming to the other cell.

In vitro, naive T cells from mice with two congenic backgrounds (either CD45.1 or CD45.2) were differentiated in vitro into all the subsets (Th1, Th2, iTreg, and Th17) for 3 days. Then, cells from one subset were taken from a background and co-cultured with cells of another subset (in equal amount), differentiated in the other background, allowing to know the origin of each cell later. After two days of co-culture in fresh medium, without additional cytokine nor TCR stimulations, the cells were assessed for cytokine production by PMA-Ionomycin restimulation and cytokine staining (Figure 4.4).

For instance, when Th1 from CD45.2 background were co-cultured with Th2 cells from CD45.1 background, 33.2 % of the Th1 cells expressed IFN-γ while the Th2 did not express it (Figure 4.4A). In the case of Gata3, the histogram of Gata3 expression was shown inside one of the populations, in dependence on the other subsets that were present in the co-culture (Figure 4.4B).

Th1 cells contained the same percent of IFN-γ producers, independently of the Th2, iTreg or Th17 cells present in the culture (Figure 4.4A). However, they were slightly increased when Th1 were co-cultured with Th1, suggesting that Th1 cells are insensitive to the signals produced by other subsets (without TCR stimulation), but receive a positive feedback from other Th1 cells.

Cells from Th2 and iTreg kept the memory of their Gata3 expression independently of the presence of other cells (Figure 4.4B). The expression of Foxp3 among iTreg cells

was similar when co-cultured with iTregs, Th2 or Th17 (45 to 47%) and showed a slight decrease in the presence of Th1 cells, suggesting that iTregs are sensitive to Th1 signals (Figure 4.4C). Finally, the same amount of IL-17 producers (44 to 47%) was observed in Th17 differentiated cells, independently on the well-mates (Figure 4.4D). Note that all the cells in the CD45.1 background come from the same pool of cells, while the Th17 cells from the CD45.2 background were separately differentiated, possibly explaining that they are slightly different for IL-17 expression.

Finally, it was striking to observe that, while cells from each subset kept memory of their cytokine secretion profile, they never acquired the markers of another subsets following co-culture. It shows that, in this context, without TCR stimulation, the cells are mainly insensitive to the signals produced by other differentiated cells.

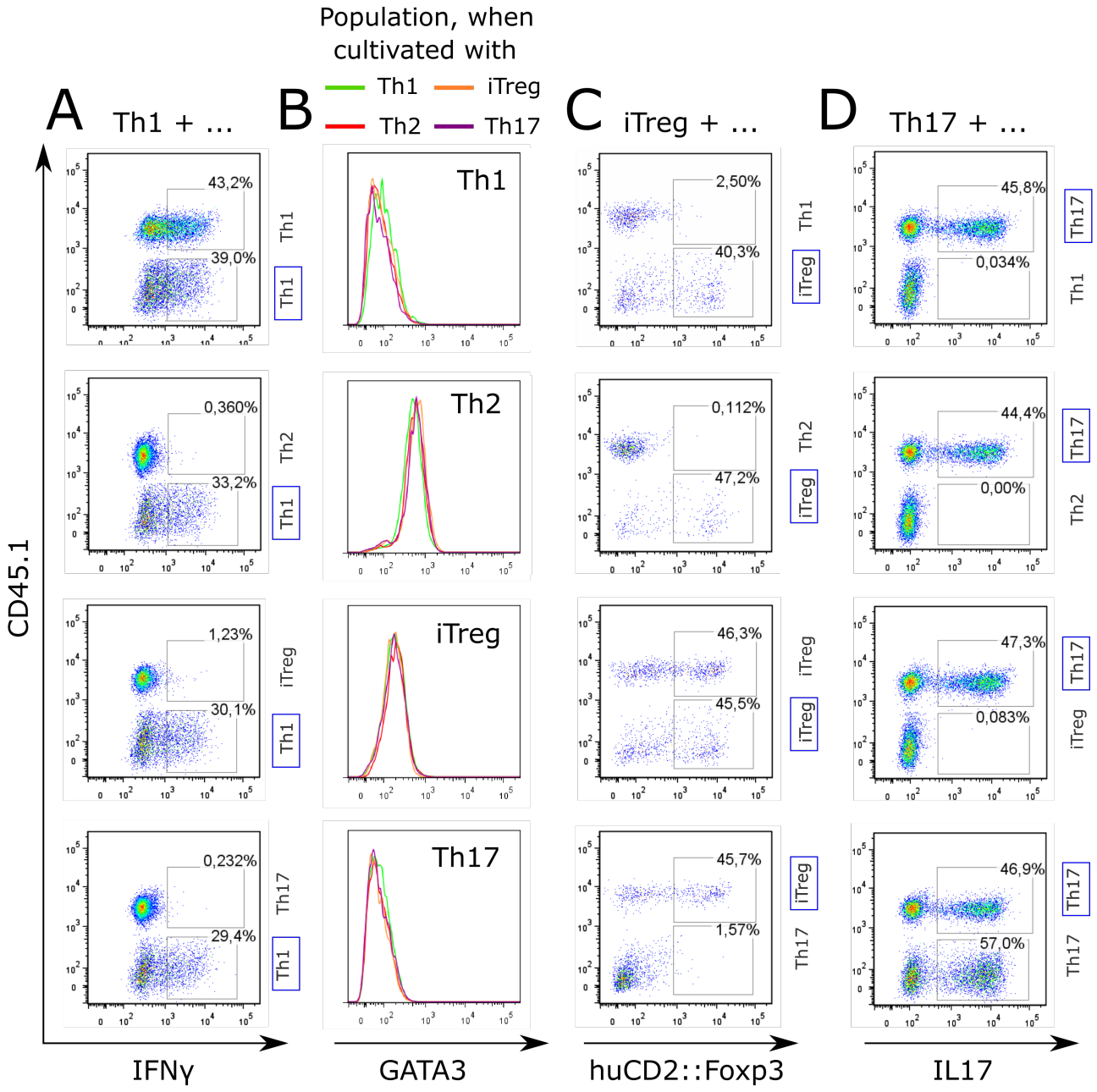
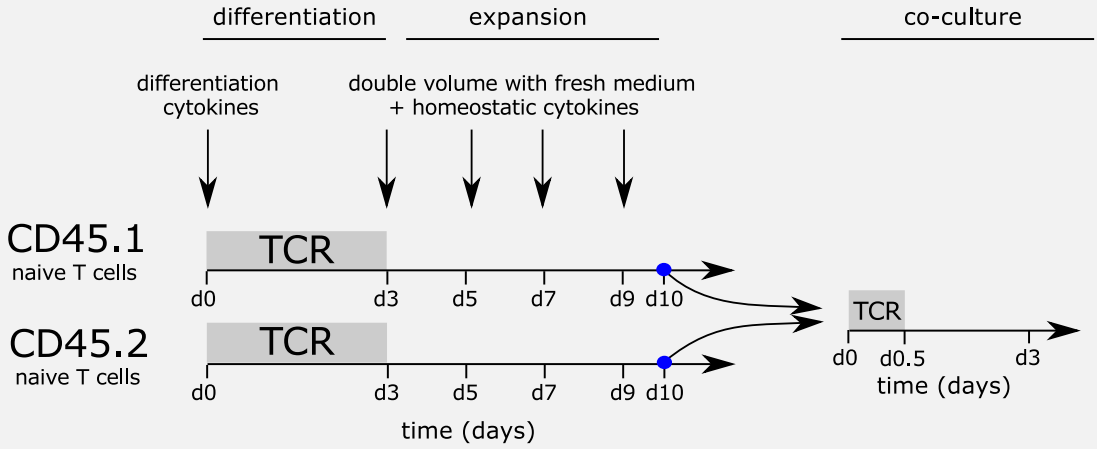


Figure 4.4: **Stability analysis upon coculture with adverse subsets.** Naive T cells from congenic mice (CD45.1 or CD45.2) were separately differentiated into all the subsets for three days. Each subset from a congenic background was further co-cultured for two days with another subset from the opposite congenic culture (in equal amount and without TCR stimulation). A,C,D: Cytokine and Foxp3 stainings are shown for different combinations of subsets, as indicated on the right of each plot. The fraction of positive cells are indicated within the congenic populations separately (CD45.1+ or CD45.1-). Note that the order was inverted in C for iTreg-Th17 co-culture. B: Level of Gata3 staining inside each subset, after co-culture with other subsets. Preliminary (performed only once).

Old cells co-restimulated keep they cytokine profile but are inhibited by well-mates

The finding that differentiated cells were stable upon co-culture without TCR stimulation was a bit surprising in light of previous findings showing that (ex vivo) Th17 cells express IFN-γR and IL-4R, and are consequently inhibited by IFN-γ [93, 237] and IL-4 [238], respectively, and these cytokines should be produced by co-cultured cells. It would be possible that, due to the lack of TCR stimulation, the cells didn't produce enough cytokines to detect an effect, or that they need TCR to be responsive to cytokine signals, through serine phosphorylation of the STATs for instance [239].

Box S10 : Experimental layout for the co-culture of old differentiated T helper subsets.



In order to avoid cell death by restimulation of young cells, naive T cells of both CD45.1 and CD45.2 backgrounds were differentiated for 3 days and expanded with fresh medium and IL-2 (or IL-23 for Th17) every 2 days for 7 additional days. Finally, cells were washed, and co-cutltured with different ratio (but the same total cell number) in anti-CD3/CD28 coated plates, in fresh medium, for 12 hours (this time period is sufficient to activate memory cells). After 12 hours the cells were replated in a normal culture plate to stop stimulation and avoid cell death, and were analysed at day 3 post activation for transcription factor expression or cytokine production by flow cytometry

Therefore, a similar experiment was performed by co-cultivating different congenic T helper subsets in vitro, but under TCR stimulations, as described in (Box S10). This experimental layout applies to (Figures 4.5, 4.6 and 4.7).

An example of cytokine secretion assay is shown in the case of Th1-Th17 cocultures, with different ratios (Figure 4.5). Only IFN-γ producers could be observed from Th1 cells (in blue), while only IL-17 producers arose from the Th17 cells, showing that the cells keep their phenotype and do not express the cytokine of the other subsets, similarly to the results in (Figure 4.4).

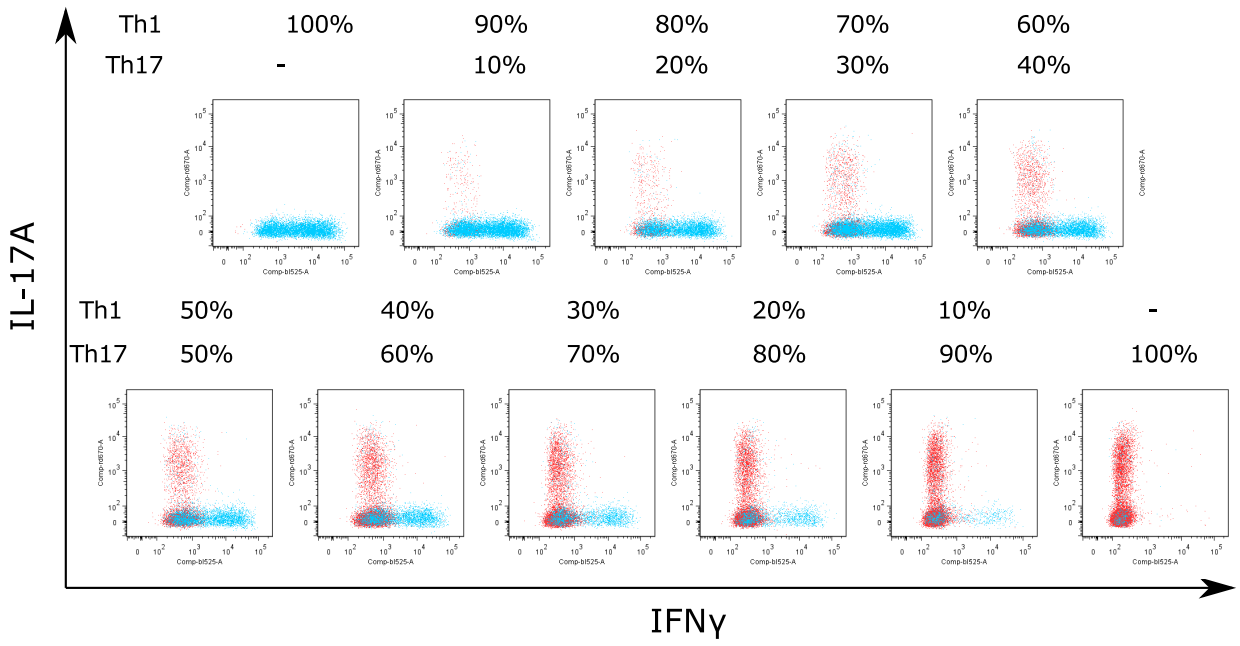


Figure 4.5: **Cytokine expression by cocultured, old, differentiated Th1 (blue) and Th17 (red) with different ratio.** Th1 cells were differentiated from CD45.1 and Th17 cells from CD45.2 background. After 10 days of expansion, they were washed, counted and restimulated with TCR for 12 hours in the same well, with different ratio. Representative of 3 different experiments.

To quantify possible inhibitions between two co-cultured subsets, the expression of cytokines or transcription factors inside one subset (separated in the analyses thanks to the congenic marker), was quantified depending on the ratio of cells from the other subset in the well. IFN-γ and IL-17 production is shown in (Figure 4.6) together with Gata3 expression in (Figure 4.7).

When Th17 cells were cultivated with Th0, Th1 or Th2 cells and with different ratio, no significant dose-dependent effect could be observed and around 30% of cells were highly producing IL-17. A decrease of IL-17 production could be observe in the presence of iTreg cells, going down to 20% when 90% of the cells in the well were iTregs. This shows that iTregs have a small suppressive effect on Th17 cells. IL-17 was never observed in non-Th17 cells.

When Th1 were co-cultured with any other subset, their production of IFN-γ diminished in a ratio-dependent manner. Th2 were very efficient in suppressing IFN-γ production, as only 10% of Th2 cells in the well were sufficient to drop the amount of high IFN-γ producers from 60% to 10%. Even Th0 cells did suppress IFN-γ production. It is not possible to separate whether a positive feedback between Th1 cells is diluted when their ratio is smaller (for the same total number of cell), or whether all the other subsets do have an inhibitory effect on IFN-γ production. Here, the co-culture with Th0 cells (which are believed to have a restricted cytokine secretion pattern), would suggest that Th1 rely on a positive feedback to support IFN-γ production. Finally, Th2 co-cultured with a high ratio started producing IFN-γ to a low extend (up to 12% of producers), showing that old Th2 are plastic to express IFN-γ, while maintaining Gata3 (see figure 4.7). Interestingly, no IFN-γ production from Th17 cells could been detected (data not shown), while Th17 have been shown to produce IFN-γ [92], suggesting that the signals leading to IFN-γ production by Th17 are independent of those produced by Th1 cells.

By looking at IL-4 production, Th2 cells lost their expression when co-cultured with cells from any other subsets, from 35% IL-4+ in a pure Th2 condition, down to 1% in conditions where they were surrounded by adverse cells. Consistently, Gata3 levels dropped down when Th2 were co-cultured with iTreg, Th2 and Th17, but not significantly with Th1 cells. It shows that, either Th2 cells rely on a positive feedback requiring a lot of Th2 cells (which is not likely as the presence of 10% of other cells strongly dampens IL-4 production), or that the other subsets secrete a factor that inhibits IL-4 production.

Strikingly, IL-4 production and high gata3 expression could be acquired by Th1 and Th17 cells when they were co-cultured with Th2 cells, likely due to the production of IL-4 by Th2

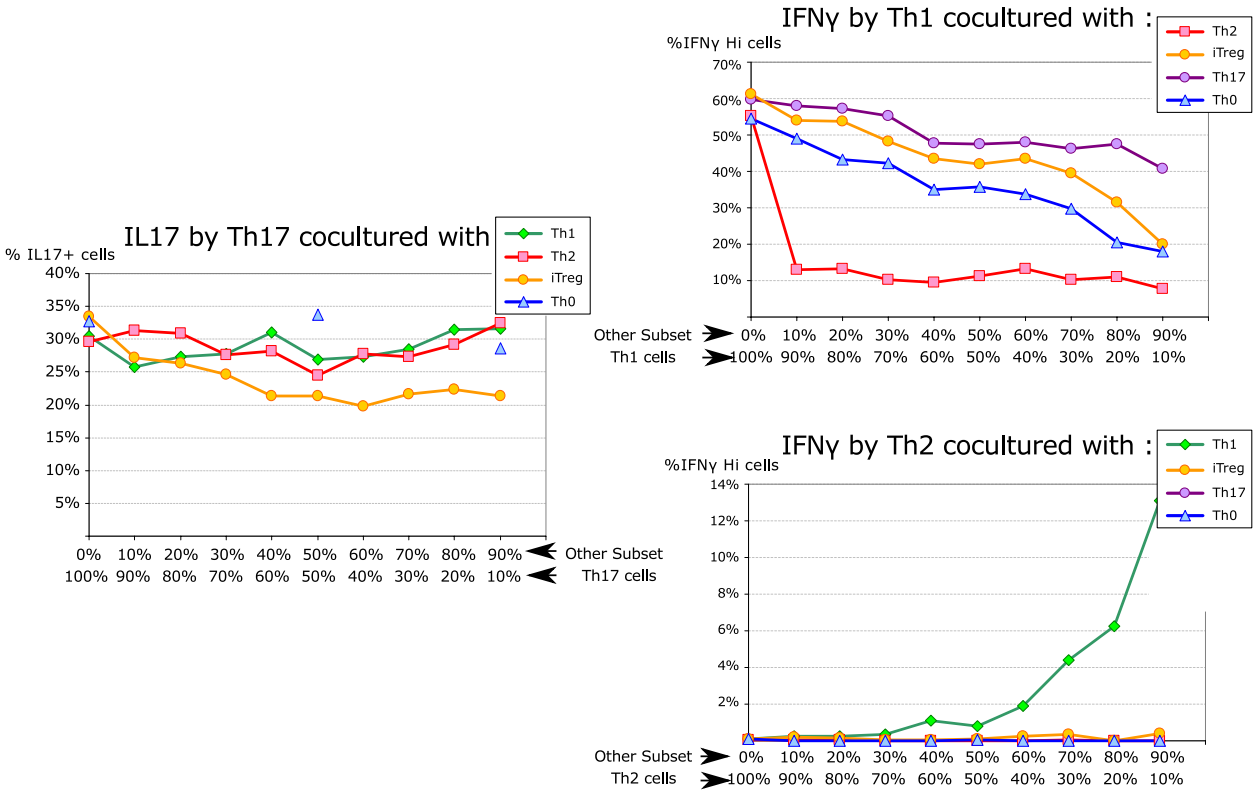


Figure 4.6: **Amount of IFN-γ and IL-17 producers among different subsets when they are co-cultured with another subset, and with different ratios.** 2 independent experiments.

cells upon TCR stimulation. Curiously, co-culture of Th17 cells with iTreg cells induced the expression of Gata3 and, to a minor extent, of IL-4, which was completely unexpected. It would be interesting to assess if these iTregs need their intermediate Gata3 expression to perform this 'Th17 suppression'.

These results show that, even if differentiated cells maintain their cytokine secretion profile upon a co-culture challenge, inhibitory mechanisms as well as acquisition of another cytokine or transcription factor could be observed.

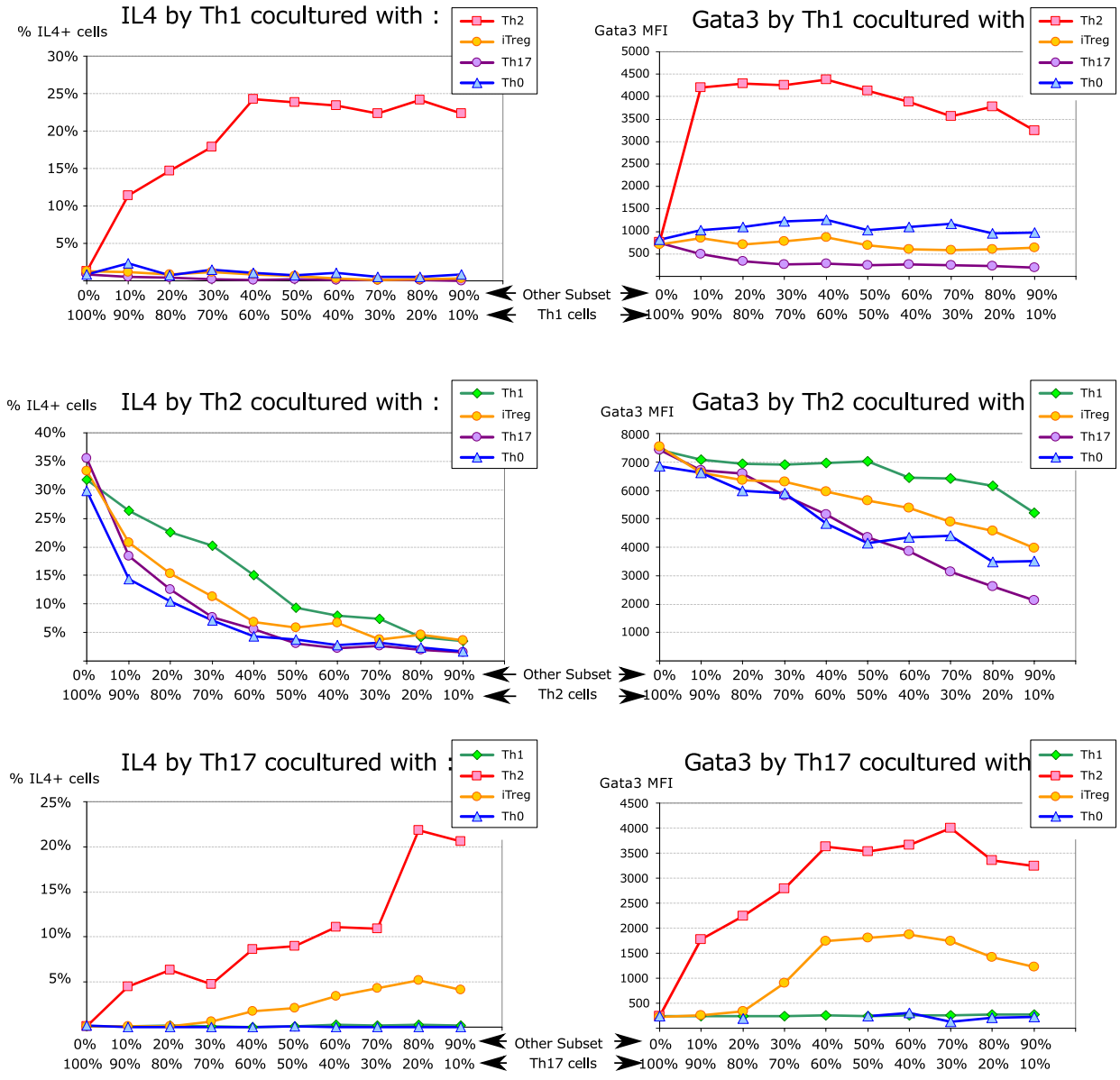


Figure 4.7: **Amount of IL-4 producers and Gata3 expression among different subsets when they are co-cultured with another subset, and with different ratios.** Preliminary (performed only once).

CHAPTER 5
DISCUSSIONS

5.1 Potential pro-inflammatory effect of Glutamine

therapeutical impact Our work highlights a critical role for extracellular nutrient availability in the terminal differentiation of CD4+ T cells to distinct effector fates. Remarkably, activation of naive CD4+ T cells under conditions of glutamine deprivation causes them to terminally differentiate into Foxp3+ regulatory T cells (Tregs). Furthermore, the skewing of glutamine-deprived naive CD4+ T cells to a Foxp3+ fate occurs even under Th1-polarizing conditions. Notably though, Th2 differentiation is not inhibited by glutamine deprivation and the cells do not adopt a Treg fate.

It suggests that the glutamine concentration and more generally, the metabolic environment could constitute a 4th signal for guiding T cell differentiation. The local response to infection is generally associated with a high metabolic activity, and an increased blood inflow. One could speculate that the presence of glutamine would skew immune cells to behave in a pro-inflammatory manner, meaning that the body could use the local metabolic state as an immune signal. Following this reasoning, a poor metabolic environment could be considered as a non-infected tissue by the immune system, and studying the heterogeneous metabolic complexity of organs could help to understand organ-specific immunity. It would be interesting to assess the immune response against the same pathogen in organs with different metabolic properties (though this might be hard because pathogens are usually organ-specific), or by modulating the local environment in vivo. The gut might be an interesting organ to follow, because of its pH gradient between the stomach and the colon [240].

Our observations as well as others would suggest to use specific diets to modulate the activity of the immune system. However, it is not sure that the observations described in the present study in vitro would apply directly in vivo, because other immune cells types could differentially respond to glutamine. Interestingly, low glutamine levels have been correlated with an anti-inflammatory immune state in vivo in humans, and glutamine administration, in specific therapeutic situations, has been shown to support the immune response, consistently with our findings:

Anaerobic training (used in sports to increase the amount of Red Blood Cells) leads to a decrease in plasma glutamine, and is associated with a decrease in CD4 T cell numbers [241]. Physical exercise has been shown to have anti-inflammatory properties, and it has been suggested to be due to a change in the amounts of glucose and glutamine in the blood [242]. Similarly, a study pinpointed that prolonged exercise induces immuno-suppression, and supplementation with a glutamine precursor reduced its severity [243]. Finally, in the case of severe infection, injections of glutamine enhanced the resolution of the pathology. [244]. In the same direction, supplementation of glutamine and leucine had a beneficial effect in the response against herpes virus [245].

The tumoral micro-environment is known to be metabolically poor, due to the consumption of nutrients by tumoral cells, and the lack of oxygen supply or blood vessels. Glutamine levels have been shown to be particularly low inside specific tumors, such as carcinomas [246] or colon tumors [247], together with glucose and other amino-acids. It raises the question whether glutamine limitation generates an anti-inflammatory shield for tumors, and would suggest to adapt the treatments in the case of glutamine-deprived tumors, to support the pro-inflammatory functions of T cells.

A potential supplementation of glutamine as an anti-tumoral treatment would need to be designed carefully because tumoral cells rely also on glutamine [248]. As an example, Elspar, a treatment that induces depletion of glutamine and arginine from the plasma, is used against a type of B cell lymphoma (ALL), and shows anti-tumor efficacy but causes immuno-depression as side effect [249]. Therefore, the respective impact of treaments on cancer cells but also on immune cells has to be carefully evaluated. One could speculate that an optimal glutamine level could be found, sufficient to boost immune cells, but low enough to maintain tumor cells under starvation. Another approach could be to instruct T cells into a different metabolic program, less dependent on glutamine. Several strategies could be envisioned. As a selection-based approach, T cells could be first differentiated in a replete environment, and progressively replace the supernatant with media with reduces

concentrations of glutamine, to select potential 'super T cells'. Another approach could be to modulate the expression of nutrient transporters or metabolic enzymes, via overexpression or the use of interfering RNA, for instance, or to find external (metabolic or cytokinic) signals that could modulate them.

One could also try to manipulate other sources of energy than glutamine in a way that tumor cells are impacted but not T cells. Acetate has been shown to be an important source of energy for tumors after glucose and glutamine [250]. Interestingly, T helper cells have been shown to be sensitive to acetate levels, and acetate was implied in the acetylation of the Foxp3 promoter [251].

Mechanisms linking glutamine sensing to Th differentiation An important question raised by this work is by which mechanisms glutamine levels are integrated into a fate decision. The study of metabolic sensing is quite complicated, because the pathways that sense metabolites also impact on the expression of metabolic enzymes processing other nutrients. Consequently, the indirect effects of different nutrient sensors are entangled, because they will help using other metabolites and indirectly activate other nutrient sensors. Importantly, the TCR itself activates many nutrient sensors. Personally, I would say it is a way to force the cell to use specific nutrients and to proliferate blindly with maximal power by thinking the metabolic environment is full of nutrients, while these nutrient sensors might not have been activated otherwise.

Several pathways have been studied to be important for metabolic reprogramming and could be responsible for the phenotype of differentiation observed under glutamine deprivation in Chapter 2:

- mTOR (mTORC1 and mTORC 2). These two complexes are amino acid and growth factor sensors, and in turn promote growth and proliferation by increasing ribosome biogenesis and translation for instance. It has recently been shown that TCR signaling upregulates ASCT2 (glutamine receptor), which in turn is necessary for glutamine entry and activation of the mTOR pathway [204]. The signals inducing mTOR in T cells are discussed in [252]. Th1 and Th17 rely in mTORC1 activation downstream Rheb [253], which is itself activated downstream amino-acid sensing. Glutamine deprivation would cause a failure to activate mTORC1 and to support Th1 and Th17 differentiation, consistently with our findings, and similarly to [204]. Regarding iTreg differentiation, the use of rapamycin, an inhibitor of mTORC1, enhanced Foxp3 expression [254], while activation of mTOR inhibited iTreg differentiation [255], consistent with

the upregulation of Foxp3 without glutamine. Therefore, mTOR could contribute to the glutamine deprivation-induced redifferentiation.

- ERK(1 and 2): ERK phosphorylation promotes proliferation and cell growth, especially for the G1/S transition of the cell cycle. In T cells, ERK is activated through the TCR signaling and by several cytokines. Interestingly, the level of ERK phosphorylation was reduced when T cells were activated in absence of glutamine [198]. Furthermore, ERK inhibition by the use of inhibitors [256] impaired Th17 differentiation while increasing iTreg differentiation. Similarly, ERK2 deficient cells showed a strongly reduced Th1 differentiation in vitro and lower T-bet expression, while Th2 differentiation was slightly enhanced and Gata3 expression was increased. Th17 was not impaired in this case (probably because only ERK2 was deleted), but the iTreg differentiation was increased at day 5. Therefore, an inhibition of ERK due to glutamine deprivation would recapitulate our observation that Th1 differentiation is inhibited at the benefit of Foxp3 expression. It is interesting to note that TGF-β can inhibit TCR-induced ERK signaling [257, 258], though TGF-β can also increase ERK activation from non-canonical pathways [132, 259], and that ERK itself modulates the functional effects of TGF-β on T cells gunnlaugsdottir2005anti. Therefore, it would be insightful to assess the dose-dependence cross-talk between TGF-β and glutamine levels on ERK and iTreg differentiation.

Other energy sensors that are not directly linked to glutamine so far, could be affected by the metabolic adaptation of the cell under glutamine deprivation, and in turn could be responsible of the impaired differentiation profile.

- AMPK can sense the energetic status of T cells, and is activated under low amounts of ATP (which we observed under glutamine deprivation), and can, in turn, downregulate mTOR. AMPK was shown to be essential for Th1 and Th17 differentiation [260]. Therefore, AMPK could also be responsible for the impairment of Th1 and Th17 differentiation via controlling mTOR. However, as AMPK is also upregulated in iTreg, a downregulation of AMPK could also impact on Foxp3 expression.

- GAPDH, an enzyme involved in glycolysis, can also bind to the 3'UTR of IFN-γ mRNA and suppress its translation when glycolysis is low [188]. It could contribute to the decrease Th1 differentiation under low metabolic activity under glutamine deprivation.

- Additional metabolic sensors such as Foxo1/Foxo3 [261], PPARγ, a sensor of lipids [262], have been involved in T helper differentiation, and are reviewed in [263].

Finally, for the beauty of complexity, these signaling pathways are interrelated, with positive and negative feedbacks, making it very difficult to investigate the effect of one of them separately [264] (Figure 5.1).

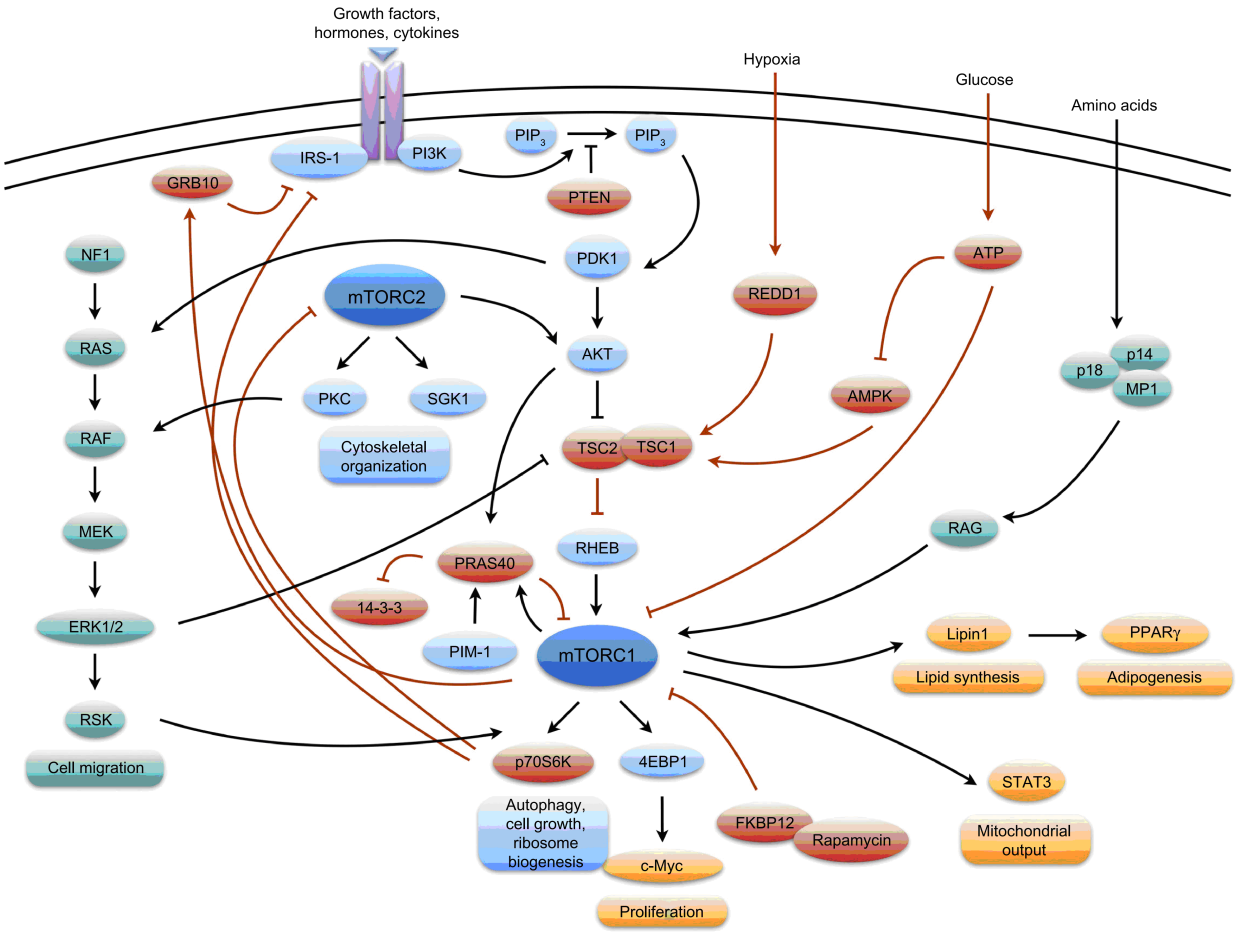


Figure 5.1: **An example of the entanglement between metabolic sensors.** Connections between mTORC1/mTORC2, AMPK, PPARγ and cMyc. Taken from [264].

In further work from the lab, we demonstrated that supplementation with a cell-permeable α-KG (a downstream metabolite of the glutaminolysis pathway) could restore an efficient Th1 differentiation under glutamine deprivation [203]. Furthermore, the phosphorylation level of S6, one of mTORC1 target, was also increased. Indeed, α-KG has been shown to activate mTOR [265] even after inhibition of glutaminolysis. These findings support, that the conversion of glutamine to α-KG, potentially regulated by the mTOR pathway, is important for terminal Th1 differentiation, Finally, mTOR has been shown to induce Glut1 expression [266], which could explain the limited proliferation of the cells actived under glutamine deprivation but in presence of glucose.

I personally think that the metabolic reprogramming of T cell activated under deprived glutamine conditions would skew many metabolic pathways into a 'low energy' profile and therefore impact all previously mentioned metabolic sensors, independently of potential cross-talks between them. Then, I would not be surprised if many synergistic pathways exist in parallel between these metabolic sensors and the expression of transcription factors such as Foxp3. It would be interesting on one side to discriminate the 'sensor profile' of T cells in response to specific nutrient modulations, and on the other side to investigate the relative effect of each sensor separately on differentiation.

The complexity of nutrient sensing pathways and the fact that all the differentiation programs were impacted by glutamine deprivation, led us to develop the mathematical model presented in chapter 3, that could be further used to predict which pathway is the most likely to influence, or is the most strongly influencing differentiation under glutamine-deprived conditions. More precisely, by assuming that one pathway has more effect on differentiation than others, the model could be used to predict which single modifications of the differentiation network could explain the phenotype observed in the context of glutamine deprivation. It would then allow to predict the efficacy of specific inhibitors in a quantitative way, including the effect of the complex regulatory network deciding for T cell differentiation in the prediction. We didn't reach this point here, but we managed to draw a mathematical model that recapitulates the kinetics of T cell differentiation. We hope that this model will be a good basis to further incorporate metabolic pathways into it.

5.2 Combining an in vitro and in silico approaches to capture the dynamical properties of T helper differentiation

The dynamics of transcription factors and cytokine expression suggest refinements of the 'master transcription factors' (MTF) paradigm The dynamics of the MTFs (T-bet, Gata3, Foxp3 and Rorγt) were followed during helper T cell differentiation in vitro, in a replete metabolic environment. It showed that some MTFs are expressed in other subsets than their respective ones, but are kept under control.

They are acknowledged as the MTFs for their respective subsets as they were shown to be necessary (i.e. their deficiency strongly abrogated the subset differentiation in vitro and in vivo), and sufficient (their overexpression was enough to induce subset-specific cytokines or functions) for the differentiation of their subset. The present study would suggest some refinements to this paradigm:

1. **The expression of the MTFs is not specific to a subset, but rather their high and sustained expression.** First, we observed, consistently with published data, [102] [100], that a MTF is not expressed in only one subset. For instance T-bet and Gata3 were expressed at intermediate levels in iTreg and Th0, and Rorγt was transiently expressed during iTreg differentiation. The intermediate expression of Gata3 was not enough to endorse with a Th2 phenotype (in iTreg or Th0), but instead, one could speculate that a threshold in Gata3 should be overcome to properly confer a Th2 phenotype. Therefore, (low) expression of a MTF alone is not sufficient, and high amounts are likely to be necessary.

2. **The (intermediate) expression of MTFs is not sufficient.** Secondly, T-bet was very highly expressed in Th0 and iTreg conditions, though transiently, without leading to high IFN-γ production. It shows that early high expression of T-bet is not enough to confer a Th1 phenotype (defined by IFN-γ production). The mathematical model could explain this observation by the synergy between T-bet and the positive feedback from IFN-γ on itself, which started earlier in Th1 condition. Biologically speaking, the specificity of IFN-γ production in Th1 could also arise from different mechanisms, for instance if T-bet has to be sustained for a sufficient period of time to allow for IFN-γ production, or if Gata3 has to be sufficiently dampened downstream IL-12 to allow

for IFN-γ production. Our data suggests that T-bet alone does not manage to provide a full Th1 differentiation, and that exogenous IL-12 provides important signals for differentiation, though other cytokines could do the same job in vivo such as IL-18.

Initial studies showed that T-bet overexpression is able to endorse T cells with the production of IFN-γ [114]. In these settings, the cells were transfected after several days of activation, and a artificially high level of T-bet was likely induced. The time-point of study is also different. In our hands, T-bet expression between 20 hours and 60 hours was not inducing IFN-γ in Th0 and iTreg conditions, but was further downregulated. It doesn't exclude that other factors could be present after day 3 that would allow this IFN-γ induction. In this case, it would be critical that T-bet is downregulated before day 3 in the Th0 and iTreg conditions to avoid the ectopic expression of IFN-γ. It would be interesting to modulate T-bet expression at late time-points to test this hypothesis.

3. **Not all the transcription factor expressing cells do produce cytokines.** In an in vitro culture, it is commonly acknowledged that not all the cells reach the state of cytokine producer. However, they all express the associated transcription factor to very high levels. Indeed, by comparing (Box S1) and (Figure 3.1), in a typical in vitro differentiation all the cells are positive for T-bet, Gata3 or Rorγt in the respective subsets, but only a few of them are reaching the state of producing the associated cytokines under PMA-Ionomycin restimulation (Box S1). Should the cells that are not producing the cytokine be considered as properly differentiated cells or not ?

4. **The concept of MTF is conceptually not unique.** Third, the notion of 'necessary and sufficient' for MTFs is conceptually not unique: If a MTF F would require to be in complex with another factor C for inducing the effector functions of a subset, and if this factor C can induce the expression of the MTF F, then both of them become necessary and sufficient. Indeed, in a deficient background for C, the complex would not form, and overexpressing C would also induce the master factor F and successfully form the complex.

   In the case of Th1, Hlx would be a candidate to the T-bet co-factors list, as Hlx overexpression induces IFN-γ production even under Th2 polarizing condition [267], while Hlx haplo-insufficiency impacted the Th1/Th2 ratio in vitro [268]. Hlx induces T-bet [269], thus making up the aforementioned connection of C and F. The effect of Hlx deficiency on T helper differentiation could not be assessed yet because it is embryonic

lethal [270]. Overexpression of T-bet in combination with Hlx in Stat4-/- Th2 cells led to an increase amount of IFN-γ producers compared to T-bet overexpression alone [269], suggesting that Hlx performs complementary functions with T-bet. It would remain to prove that T-bet alone, in a Hlx knock down context, would not be able to induce IFN-γ, to include Hlx as an other MTFs for the Th1 subset. In the present study, the presence of a second factor in Th1 could help to explain why Th0 or iTregs highly expressing T-bet are not successful into producing IFN-γ, as this second factor could be IL-12 induced (like Hlx [271]) or generally speaking Th1 specific. It would be interesting to measure Hlx in parallel with T-bet during differentiation.

5. **MTFs work in groups** The MTFs have been shown to make complexes with several transcription factors, which, in turn, determine their transcriptional capacity, and the target genes they bind. For instance, T-bet can bind Gata3 and inhibit the activation of Th2 genes by Gata3 [272]; Foxp3 can bind Rorγt and inhibit IL17 expression [159]. Similar effects (positive or negative) can be observed by direct binding with the Runx proteins [273, 274]. In [109], genome-wide binding of Th17 specific genes (STAT3, IRF4, BATF, c-Maf, and RORγt) was investigated and showed that they co-localize in most binding sites, suggesting that RORγt functions in a complex, whose composition will determine the binding sites and activated genes.

   It raises the questions: Which complexes are important for T helper differentiation and which factors of these complexes have a quantitative effect ? and how is a gene regulated in dependence on the amount of each protein involved in the complex ? Our modeling work suggest that, inside the potential transcriptional complexes, the MTFs are the ones quantitatively deciding for the transcription, because the model could explain the kinetics of differentiation only by including the amount of the master factors. In case that multiple factors have a quantitative effect, this question is very tricky because of the gigantic amount of data required to understand the quantitative response of a promoter to many input factors. One could speculate that the amount of complexes successfully formed could be directly determined by the limiting factor in the complex, and would allow a simple mathematical formulation.

implications of the observed latencies in vitro Another result from this combined in vitro and in silico approach is the identification of major delays (or saturation effects). Delays in Foxp3 transcription, in translation and cytokine secretion were necessary to explain the link between mRNA and protein kinetics, and opens further speculations:

First, on the methodological level, most modeling studies rely only on one level of data (most of the time being transcriptomics). By neglecting the dynamics of the protein levels, it becomes very hard to reveal a correlation in time between activators and activated genes because they are separated in time. Without delays, the real network would probably not fit the data, while wrong networks would succeed in explaining the data (by using other activators or inhibitors to artificially reproduce the dynamics of the genes expression). It is an open question to know if mRNA networks with delays are adequate tools to capture the latencies. It would be interesting to generate synthetic data from a bi-level mRNA-protein network, and to see if a similar model at the mRNA level only would be able to detect and reproduce those delays.

Second, the term delay is not necessarily accurate, as we don't know the mechanisms responsible for the observed latencies. They might be due to additional activators or inhibitors that require time to be expressed or dampened; it could be due to the storage of mRNA molecules to be transcribed later, as described in NK cells [275]; a regulation in translation, or a regulation by the cell cycle. In our work, we considered these delays as a saturation in the translational and secretion machinery, with a specific time-scale, independent on external signals. However, it is still possible that cytokine or TCR signals have an impact on the time-scales of the delays, in which case a modulation of the TCR strength could impact differentiation by allowing an earlier cytokine secretion. Additionally, when switching the differentiation condition at 20 hours in (Figures 4.2, 4.1 and 4.5), the delays might be re-initiated and could explain why the model was not completely reproducing the experimental data. In the model, while the translation and secretion capacity should not be impacted by switching, a 'reprogramming transcriptional latency' for the promoters of Rorγt and Foxp3 could be included, or an epigenetic mechanism could be explicitly modelled.

The delay in translation has interesting consequences. Peaks in mRNA could be observed that were not converted into proteins (or to only minor levels), as for T-bet in the first hours (Figure 3.1). This mechanism might give differentiating cells a certain level of plasticity, where the cell has a certain time-frame with limited memory to decide what will be its differentiation profile. During this time, the cell might integrate signals coming from different APCs, and an initial T-bet peak might be dampened by further opposite signals. It would be interesting to simulate and perform experiments with repeated stimulations of the cells with different cytokines, to see how the decision is taken at the end of this time-window. The latency in cytokine secretion is consistent with previously published observations that Th1 and Th2 differentiating cells usually need to trigger the first division to produce cytokines

[276] (though this is not always the case for Th17 cells, data not shown).

Where is the limit between plasticity and stability ? Finally, the last chapter shows that T cells tend to keep their cytokine secretion phenotype when co-cultured with other differentiated cells, while sometimes acquiring an additional phenotype. It shows that the notion of stability is more complex and quantitative than previously appreciated. It would be interesting to separate which mechanisms are responsible for the partial stability of differentiated cells. Stability could arise by different mechanisms such as: 1/ through positive feedbacks from released cytokines 2/ by modulating the cytokine receptors levels and making the cell deaf to adverse signals 3/ by inhibiting specific cytokine signaling pathways thanks to the SOCS proteins 4/ by imprinting the expression of cytokines or transcription factors as epigenetic marks. It would be interesting to assess of the positive feedbacks are strong enough to support the stability of a subset. The model could be extended with these mechanisms to investigate their consequences.

Thoughts about the mathematical modeling approach In this work, we developed a mathematical model to capture the key features of T helper differentiation, by taking the core factors and their interactions, and by developing suitable, time-resolved data. Two limitations of the mathematical model could be observed: First, only a minority of parameters were actually identifiable from the data (Appendix, Figure 7.3), and second, the model only partially reproduced the experiments where the differentiation conditions were switched at 20 hours (Figure 4.3).

Therefore, it shows that learning the properties of a network from a time-resolved data in canonical condition is not enough to fix all its interactions (even with more than 1000 data points !). It is likely that some interactions between transcription factors are not active during canonical differentiation. Indeed, in an inhibition, both factors should be expressed to significant levels to detect an effect of the inhibition. Therefore, the kinetics of canonical differentiation do not contain such information and the model could not extract them. In this case, the switching experiments will be a good additional dataset to be included in the fitting.

Another source of non-identifiability is the complexity of the equations and the number of parameters. To increase the number of identifiable parameters, a solution would be to simplify the network or the equations in a way that reduces the overall number of parameters. The functions describing transcriptional activations or inhibitions (hill functions with 3 parameters) could be simplified to 2-parameters hill functions. As a preliminary test, the

simplified equations allowed for a good fitting of the kinetics of all transcription factors but Foxp3 (not shown), meaning that Foxp3 has specific sensitivity properties to signals, and needs a more general hill function. Another simplification mechanism would be to randomly remove interactions in the network, in order to discriminate which interactions are required to fit the kinetics. However, this technique is biologically dangerous, because these interactions might be inactive during the measured canonical differentiations, but active during other experimental settings or predictions, and because the fitting method might artificially overload redundant pathways to the removed interaction. Nevertheless, a simplification of the equations would not solve the lack of information in the canonical differentiations, and would only be a complementary approach to reach a full identifiability.

It is important to define first which set of data and which behaviors a model should reproduce. In the present study, the 'training data set' was the kinetics of transcription factors and cytokines during differentiation. If the model does recapitulate a set of experimental data, one can say that this model is a good representation of this data/problem. In some sense, it means we use modeling for learning data into a structure that can, at least, reproduce this particular set of data when we need. It is just a condensed representation, but not unique, of a set of experiments. In our case, the fact that the model could recapitulate the kinetics of all factors during T cell differentiation showed that the list of hypotheses incorporated in the model are enough to explain reality, and that they are consistent with each-other.

The network representation would probably be equivalent to use any structure or learning method (a black box !) such as a neural network, and to include the topological data of known interactions as additional constraints in the training dataset. Then, whatever the structure such black box would have, as long as it matches the data, one can always make predictions with it, and they might be true ...

Then comes the question: can we trust the predictions of a model according to data it didn't see before ?

It is probably impossible to know for sure...

The aim of predictions should be to discover new things or get new understanding that suggest predictive experiments whose result will be reality. If the model allowed to discover or design new experiments that revealed unexpected findings, the model was useful, led to new experimental knowledge, and can glorily be put into pension ! In this case, we can trust the predictions if they are finally experimentally true ! If the predictions failed, new questions can emerge regarding the why, and lead to an improvement of the network.

To conclude, every model has a range of validity. The training dataset is, of course, included in this range, and, if one believes into continuity of biological systems, the model should still be true for 'close' situations than the initial training dataset.

5.3 Future work and extensions of the model

When the model will (hopefully) be able to reproduce the dataset of redifferentiation upon condition switching at 20 hours (presented in Chapter 4), several predictions could be derived and suggest validating experiments:

1. The differentiation under combination of different cytokines could be described, in order to delineate which conditions lead to a 'clean' T helper subset, and which conditions would lead to a 'mixed states' phenotype. These extreme conditions could be tested experimentally and provide a protocol to generate bi-functional T helper cells. The switching experiments from Th2 to iTreg at 20 hours (Figure 4.1) show the development of Foxp3+ cells with a high expression of Gata3, and suggests that switching experiments could be a solution to produce double positive subsets.

2. By simulating long-term differentiation, the model could help to assess if the strength of positive feedbacks can stably maintain a subset. Strikingly, preliminary simulations suggest that the expression of transcription factors decays with time, and would suggest that the stability, in long term, is not encoded by the expression of the master transcription factor alone. It could be via the regulation of cytokine receptor levels for instance, and this could be tested experimentally.

3. By including the possible mechanistic hypotheses linking glutamine sensing to differentiation, the model could help to separate whether the results from Chapter 2 can be explained by mainly one pathway or not.

4. The model could be used to predict optimal redifferentiation protocols, from a fully differentiated cell to another subset, or to describe in which cases plasticity can occur.

5. The scanning of APCs by T helper cells might lead to contradicting signals over time. The model could simulate the encounter with several APCs providing a heterogeneity of signals, and to describe what signal patterns are necessary for a proper Th1 differentiation for instance. It would be a 'signal integration model' for cytokine signals during T helper differentiation, similarly for the model for thymic selection that we

developed in [277]. The impact of the latencies in the first hours, when mRNA is not translated into protein, could be assessed, and a potential time-windows for signal integration could be revealed.

6. It would be interesting to include this model in a population model of independent T helper cells following the same differentiation network but harboring an intrinsic variability. The expression of cytokines and Foxp3 could be probabilistic in this model (all-or-none expression inside each cell), allowing to directly compare a simulation with flow cytometry results. Additionally, the consequences of naive cells heterogeneity could be investigated.

As a conclusion, I hope that this model will further be used to make sense from a larger set of experimental data, and hopefully make the field of T cell differentiation a little bit simpler ...

5.4 Conclusion and impact

In this work, we showed that the metabolic environment, in the case of glutamine levels, has a dominant impact on T helper differentiation in comparison to the presence of cytokines. It suggests to develop specific diets or to plan direct injections of glutamine in combination of classical therapies in order to further modulate the immune response.

The kinetics of T helper differentiation in vitro were followed and a mathematical model of T helper differentiation could be trained with this dataset to reproduce the experimental data. The parameter estimation was only possible thanks to the availability of both mRNA and protein levels over time, by using an iterative fitting strategy. Additionally, latencies could be observed between mRNA and protein levels. It suggests to systematically include both mRNA and protein data in the design of future modeling projects. While the model could reproduce the kinetics of the main factors during differentiation, certain inhibitions could not be estimated by the model. It means that monitoring the kinetics of the network's elements is not enough to identify all interactions between them. It would be very helpful to develop tools to estimate the predictive power of a predefined set of experiments, or to design minimalistic sets of experiments necessary to identify all the parameters. Nevertheless, the model was able to partially predict the kinetics of the transcription factors upon switching of the differentiation conditions, giving hope that it will soon be able to recapitulate this data set when it will be included as an additional training data set.

T helper differentiation is a very complex process, and involves the regulation of hundreds of genes and dozens of pathways. It is interesting to see that a very strict simplification of the biological differentiation network was able to quantitatively explain the dynamics of the master transcription factors and cytokines. I believe that this is a big step in the quantitative understanding of T helper differentiation, especially in separating which genes and pathways are most important.

T helper differentiation shows unique properties, such as a differential plasticity at early versus late time-points, and the capacity to express multiple transcription factors at the same time. Just by looking at the minimalistic differentiation network, one could already speculate about reasons that could explain these properties, and I have the personal feeling that the current mathematical model (or small extensions of it) will be able to recapitulate the most important properties of helper T cell differentiation, and assist the development of immunotherapies.

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Picture credits:

```
Figure 1:
  Bacteria: https://blogs.scientificamerican.com/guest-blog/scientists-discover-that-antimicrob
     ial-wipes-and-soaps-may-be-making-you-and-society-sick/
  Tissue of cells: https://www.sciencetopia.net/biology/stratified-compound-epithelial-tissues
Figure 5:
  Mr. Pathogen: http://www.nutters.com/old/apr11zinc.html
  T cells: http://www.eonexperience.com/eon-models/details.aspx?cid=2185#.T_8m-_MNoPE.pinterest
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CHAPTER 7
APPENDIX

7.1 Comparison of the two replicates of kinetics

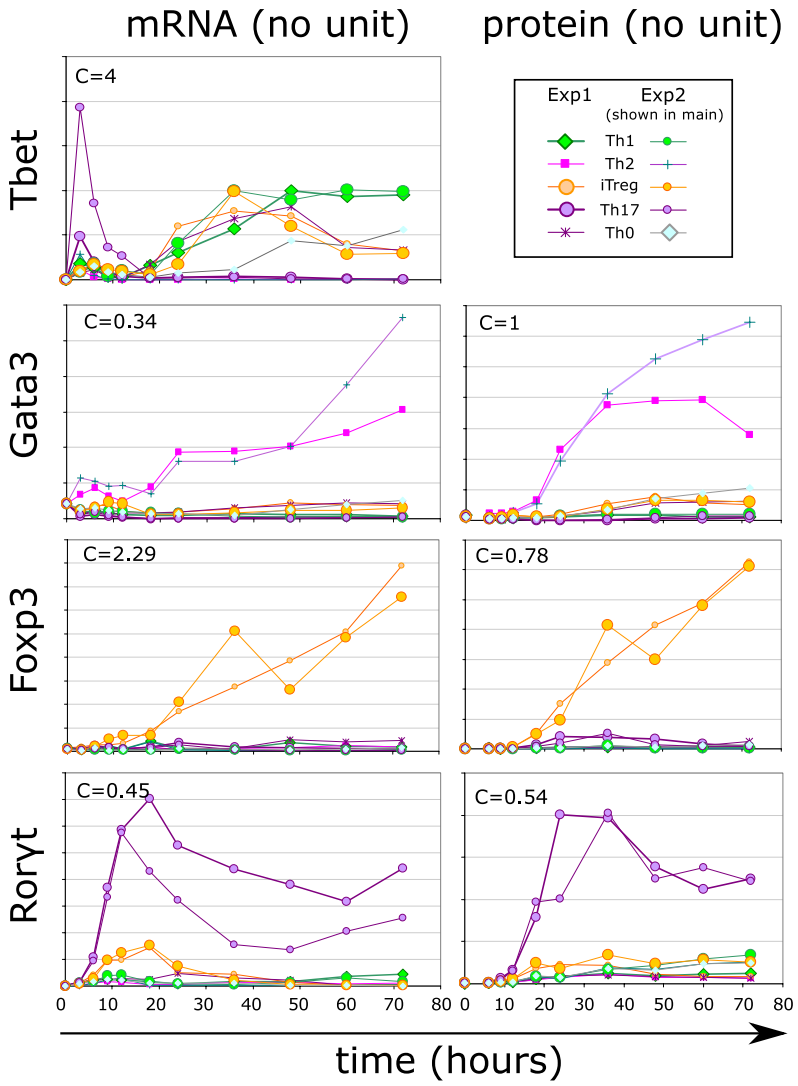

Figure 7.1: **Comparing kinetics replicates.** Among 5 kinetics realized, 2 were technically successful and are shown here. As the staining efficiency changes between experiments, one replicate had to be renormalized by a coefficient (C, shown for each graph), to be in the same scale as the other replicate. Unfortunately, the same effect appeared on the qPCR data, and the replicates had to be normalized by a coefficient again. This is unfortunate. No standard deviations are drawn because 1/ they depend on the normalization and 2/ each experiment has interesting properties that would be lost upon averaging, like the fact that IL-21 and RORγ were earlier in one replicate.

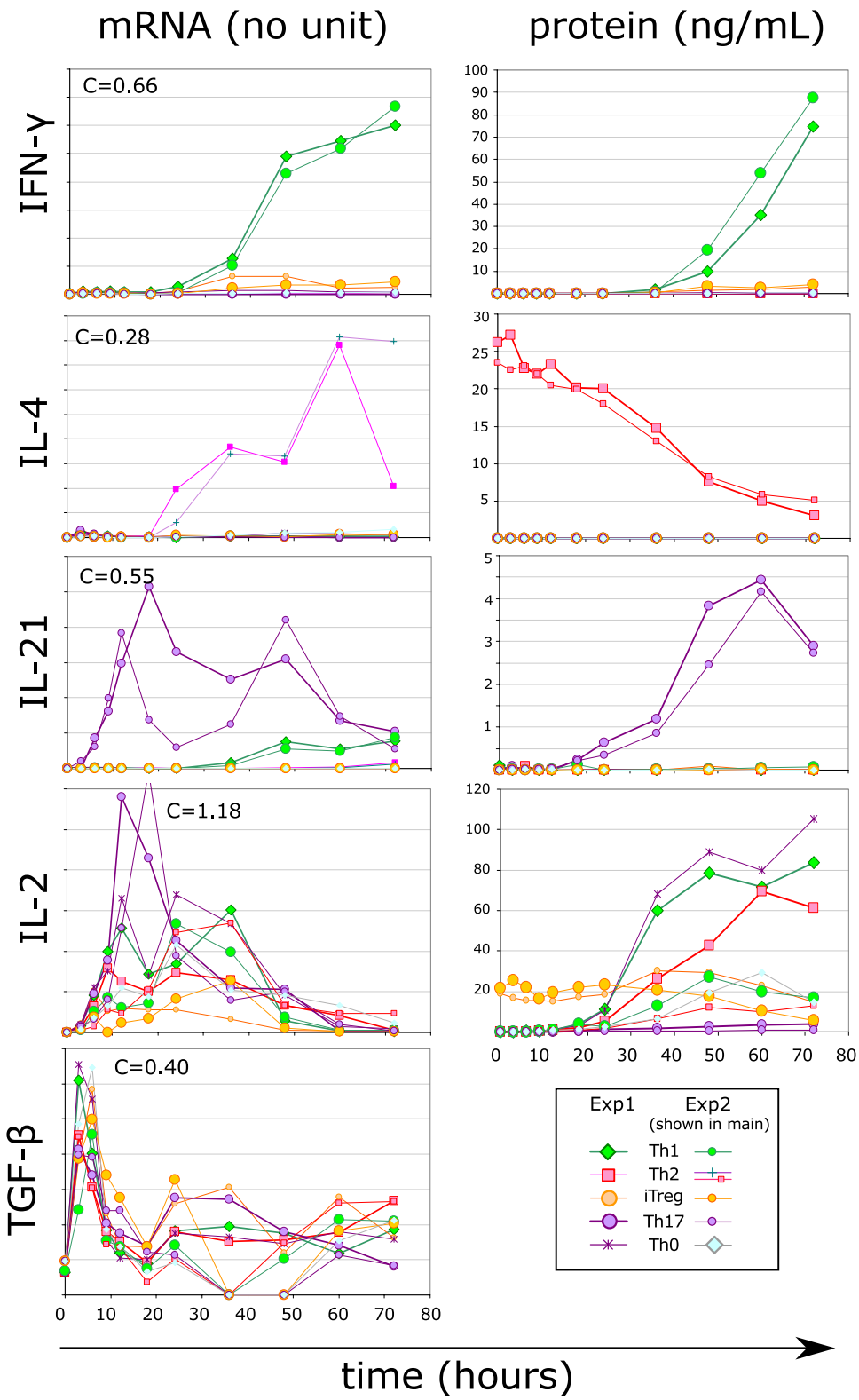

Figure 7.2: **Comparing kinetics replciates,**following of previous figure.

7.2 Parameter values and identifiability

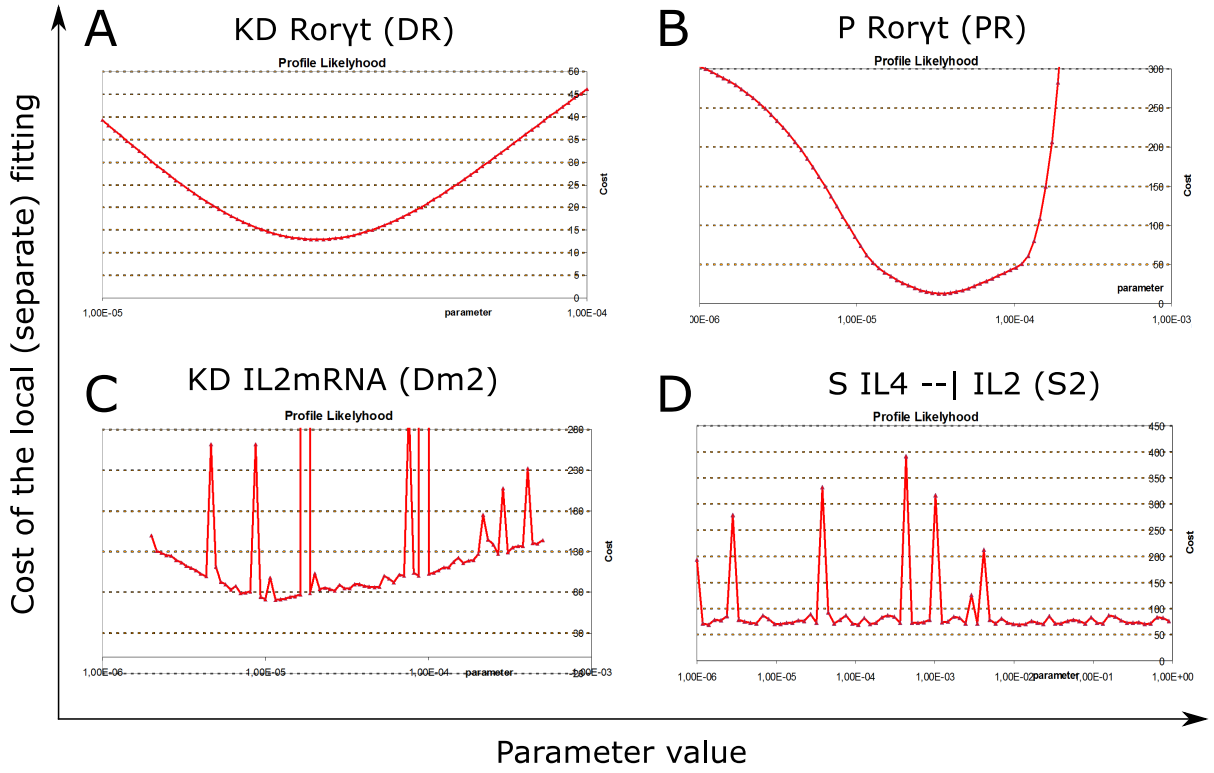

Figure 7.3: **Exemple of parameters that are identifiable or not.** The identifiability analysis for a parameter P was performed by scanning a large range of values for P, and by doing a local fitting (separate fitting as described in Box S7). The cost of the local fitting is shown, in dependence of the parameter value. The parameters $KDRor\gamma t$ (short name DR), $PRor\gamma t$ (short name PR) and $KDIL2mRNAt$ (short name Dm2) are identifiable because there is a value of the parameter that gives an optimal fitting. However, as most of the parameters, the parameter $SIL4toIL2$ (S2) is not identifiable because, whatever its value, a good fit can be obtained.

Figure 7.4: **List of parameter values, names and identifiability.** The list of parameters is shown next pages, together with their best fitted value, the range of values that was autorized for the fitting for each parameter, their short name in the equations in (Box S9), and a green tag if they are identifiable.

Parameter name Long	Short	Best Value	Boundaries for optimization Min Value	Max Value	Chosen Value	Identifiable ?
TCRPEAK		2.5			2.5	
TCRCOEFF		0.005			0.005	
KTCRGATA3	Kub	1700	1500	1700		
KTCRIL2		8500	1.1	10000		
KTCRGATA3POS		200	100	10000		
KTCRTGFB		1000			1000	
KTCRTBET		7000	1.1	20000		
KTCRIFNG		500	1.1	2000		
KDIL2	D2	1.55731e-06	0.000001	0.0001		Yes
KDIL4	D4	7.12297e-06	0.000001	0.0001		Yes
KDIL12	D12	0.000005			0.000005	
KDIL17	D17	3.55087e-06	0.000001	0.0001		Yes
KDIL21	D21	8.1254e-06	0.0000001	0.0001		Yes
KDIFNG	Dγ	0.0000001	0.0000001	0.0001		
KDTGFB	Dβ	8.4e-06			8.4e-06	
KDTBET	DT	2.5e-05	0.00001	0.0001		Yes
KDGATA3	DG	0.00001	0.00001	0.0001		
KDRORGT	DR	2.77433e-05	0.00001	0.0001		Yes
KDFOXP3	DF	8.48888e-05	0.00001	0.0001		
KDIL2mRNA	Dm2	1.11032e-05	0.000002	0.0005		Yes
KDIL4mRNA	Dm4	0.000376032	0.000002	0.0005		
KDIL17mRNA	Dm17	1.09044e-05	0.000002	0.0005		Yes
KDIL21mRNA	Dm21	0.000243753	0.000002	0.0005		
KDIFNGmRNA	Dmγ	6.38441e-05	0.000002	0.0005		Yes
KDTGFBmRNA	Dmβ	0.000364359	0.000002	0.0005		Yes
KDTBETmRNA	DmT	0.0003	0.000002	0.0005		
KDGATA3mRNA	DmG	0.00007	0.000002	0.0005		
KDRORGTmRNA	DmR	1.27605e-05	0.000002	0.0005		
KDFOXP3mRNA	DmF	1.46476e-05	0.000002	0.0005		
PIL2	P2	0.000283701	0.000001	0.001		
PIL4	P4	0.000001	0.000001	0.001		
PIL17	P17	0.000377915	0.000001	0.001		
PIL21	P21	9.17272e-06	0.000001	0.001		
PIFNG	Pγ	0.000006	0.000001	0.001		
PTGFB	Pβ	3.5e-07			3.5e-07	
PTBET	PT	2.8e-05	0.000001	0.001		
PGATA3	PG	1.5e-05	0.000001	0.001		
PRORGT	PR	3.46246e-05	0.000001	0.001		
PFOXP3	PF	0.000103757	0.000001	0.001		
CTGFBmRNA	Cβ	0.00025	0.00001	1		Yes
CIL2mRNA	C2	2.0579e-05	0.00001	1		
SIL2_TO_IL2mRNA	S1	0.0013414	0.000001	0.9		
KIL2_TO_IL2mRNA	K1	0.760337	0.01	50		
NIL2_TO_IL2mRNA	N1	0.584518	0.25	5		
SIL4_TO_IL2mRNA	S2	0.436039	0.000001	0.9		
KIL4_TO_IL2mRNA	K2	6.36339	1	20		
NIL4_TO_IL2mRNA	N2	2.01114	0.25	5		
CIL4mRNA	C4	6.49118e-05	0.000001	1		
SGATA3_TO_IL4mRNA	S3	53.5431	1.1	50		
KGATA3_TO_IL4mRNA	K3	9.07549	0.01	10		
NGATA3_TO_IL4mRNA	N3	3.05248	0.25	5		
CIL17mRNA	C17	2.38623e-06	0.000001	1		
SRORGT_TO_IL17mRNA	S3	57.2673	1.1	50		
KRORGT_TO_IL17mRNA	K3	7.54232	0.01	10		Yes
NRORGT_TO_IL17mRNA	N3	4.62537	0.25	5		
CIL21mRNA	C21	5.69316e-05	0.00001	1		
FIL6_TO_IL21mRNA	S4	19.7021	1.1	50		
SRORGT_TO_IL21mRNA	S5	2.19418	1.1	50		Yes
KRORGT_TO_IL21mRNA	K5	0.367529	0.01	10		
NRORGT_TO_IL21mRNA	N5	4.95503	0.25	5		
CIFNGmRNA	Cγ	8.80296e-06	0.00001	1		
FIL12_TO_IFNGmRNA	S6	3.57639	1.1	100		Yes
STBET_TO_IFNGmRNA	S7	19.7651	1.1	250		Yes
KTBET_TO_IFNGmRNA	K7	7.54701	0.01	10		
NTBET_TO_IFNGmRNA	N7	4.9046	0.25	5		

Parameter name Long	Short	Best Value	Boundaries for optimization Min Value	Max Value	Chosen Value	Identifiable ?
CTBETmRNA	CT	7.4e-06	0.000005	1		
FIL6_TO_TBETmRNA	S8	4	1.1	50		
SIFNG_TO_TBETmRNA	S9	370	1.1	500		
KIFNG_TO_TBETmRNA	K9	0.005	0.0001	100		
NIFNG_TO_TBETmRNA	N9	1.3	0.25	5		
SGATA3_TO_TBETmRNA	S10	0.08	0.000001	0.9		
KGATA3_TO_TBETmRNA	K10	1.1	0.01	10		
NGATA3_TO_TBETmRNA	N10	4	0.25	5		
SRORGT_TO_TBETmRNA	S11	1			1	
KRORGT_TO_TBETmRNA	K11	1			1	
NRORGT_TO_TBETmRNA	N11	1			1	
SFOXP3_TO_TBETmRNA	S12	1			1	
KFOXP3_TO_TBETmRNA	K12	1			1	
NFOXP3_TO_TBETmRNA	N12	1			1	
CGATA3mRNA	CG	0.000005	0.0000001	1		
FIL12_TO_GATA3mRNA	S13	0.0754729	0.001	1		
SIL2_TO_GATA3mRNA	S14	15	**1.01**	50		
KIL2_TO_GATA3mRNA	K14	10	0.01	100		
NIL2_TO_GATA3mRNA	N14	1	0.25	5		
SIL4_TO_GATA3mRNA	S15	6.7	1.1	50		
KIL4_TO_GATA3mRNA	K15	1	0.01	20		
NIL4_TO_GATA3mRNA	N15	5	0.25	5		
STBET_TO_GATA3mRNA	S16	0.5	0.000001	0.9		
KTBET_TO_GATA3mRNA	K16	9.80531	0.01	10		
NTBET_TO_GATA3mRNA	N16	4.97157	0.25	5		
SGATA3_TO_GATA3mRNA	S17	10	**1.01**	50		
KGATA3_TO_GATA3mRNA	K17	0.9	0.01	10		
NGATA3_TO_GATA3mRNA	N17	5	0.25	5		
SRORGT_TO_GATA3mRNA	S18	1.19371e-05	0.000001	0.9		
KRORGT_TO_GATA3mRNA	K18	3.47832	0.01	10		
NRORGT_TO_GATA3mRNA	N18	1.50898	0.25	5		
SFOXP3_TO_GATA3mRNA	S19	1			1	
KFOXP3_TO_GATA3mRNA	K19	1			1	
NFOXP3_TO_GATA3mRNA	N19	1			1	
CRORGTmRNA	CG	1.07493e-05	0.000001	1		
FIL6_TO_RORGTmRNA	S20	7.31	1.1	50		
SIL21_TO_RORGTmRNA	S21	1.9952	1.1	50		
KIL21_TO_RORGTmRNA	K21	0.256801	0.01	5		
NIL21_TO_RORGTmRNA	N21	0.464489	0.25	5		
STGFB_TO_RORGTmRNA	S22	30.4449	1.1	50		
KTGFB_TO_RORGTmRNA	K22	0.193608	0.01	1		
NTGFB_TO_RORGTmRNA	N22	0.338004	0.25	5		
STBET_TO_RORGTmRNA	S23	0.166806	0.000001	0.9		
KTBET_TO_RORGTmRNA	K23	1.89016	0.01	10		
NTBET_TO_RORGTmRNA	N23	0.313054	0.25	5		
SGATA3_TO_RORGTmRNA	S24	0.00648032	0.000001	0.9		
KGATA3_TO_RORGTmRNA	K24	1.24152	0.01	10		
NGATA3_TO_RORGTmRNA	N24	2.05171	0.25	5		
SFOXP3_TO_RORGTmRNA	S25	0.0393515	0.000001	0.9		
KFOXP3_TO_RORGTmRNA	K25	0.0590501	0.01	10		
NFOXP3_TO_RORGTmRNA	N25	2.96369	0.25	5		
CFOXP3mRNA	CF	3.1247e-05	0.00001	1		
SIL2_TO_FOXP3mRNA	S26	1.5187	1.1	50		
KIL2_TO_FOXP3mRNA	K26	30.9185	0.01	100		
NIL2_TO_FOXP3mRNA	N26	0.36997	0.25	5		
STGFB_TO_FOXP3mRNA	S27	34.4938	1.1	50		
KTGFB_TO_FOXP3mRNA	K27	0.0813677	0.01	1		
NTGFB_TO_FOXP3mRNA	N27	6.96065	0.25	5		
STBET_TO_FOXP3mRNA	S28	0.00133925	0.000001	0.9		
KTBET_TO_FOXP3mRNA	K28	0.29739	0.01	10		
NTBET_TO_FOXP3mRNA	N28	0.413513	0.25	5		
SGATA3_TO_FOXP3mRNA	S29	7.08221e-06	0.000001	0.9		
KGATA3_TO_FOXP3mRNA	K29	5.14001	0.01	10		
NGATA3_TO_FOXP3mRNA	N29	5.17163	0.25	5		
SRORGT_TO_FOXP3mRNA	S30	0.00017402	0.000001	0.9		
KRORGT_TO_FOXP3mRNA	K30	0.528483	0.01	10		
NRORGT_TO_FOXP3mRNA	N30	1.44957	0.25	5		

Parameter name Long	Short	Best Value	Boundaries for optimization Min Value	Max Value	Chosen Value	Identifiable ?
IL2EQ		0.00001	Initial values for each variables		0.00001	
IL4EQ		0.00001			0.00001	
IL12EQ		0.00001			0.00001	
IL17EQ		0.00001			0.00001	
IL21EQ		0.00001			0.00001	
IFNGEQ		0.00001			0.00001	
TGFBEQ		0.00001			0.00001	
TBETEQ		0.00001			0.00001	
GATA3EQ		0.416			0.416	
RORGTEQ		0.000001			0.000001	
FOXP3EQ		0.04			0.04	
IL2mRNAEQ		0.0094			0.0094	
IL4mRNAEQ		0.0165			0.0165	
IL17mRNAEQ		0.00016			0.00016	
IL21mRNAEQ		0.034			0.034	
IFNGmRNAEQ		0.041			0.041	
TGFBmRNAEQ		0.97			0.97	
TBETmRNAEQ		0.015			0.015	
GATA3mRNAEQ		1.36			1.36	
RORGTmRNAEQ		0.032			0.032	
FOXP3mRNAEQ		0.213			0.213	
IL4INITTH2		14.6			14.6	
IL2INITTREG		19.05			19.05	
FORCEIL2	F2	0.0006	Delays		0.0006	
FORCEIL21	F21	0.00053			0.00053	
FORCEFOXP3	Ffp3	0.00014			0.00014	
FORCERORGT	Fror	0.00042			0.00042	
FORCETRANSL	Ft	0.00014			0.00014	
FORCESECRET	Fs	0.000112			0.000112	
KANTI_IL2	BaIL2	0.000001			0.000001	
KANTI_IFNG	BaIFNg	0.000001			0.000001	
KANTI_IL4	BaIL4	0.000001			0.000001	

7.3 Side comparison of our data with a Th0/Th17 published kinetics

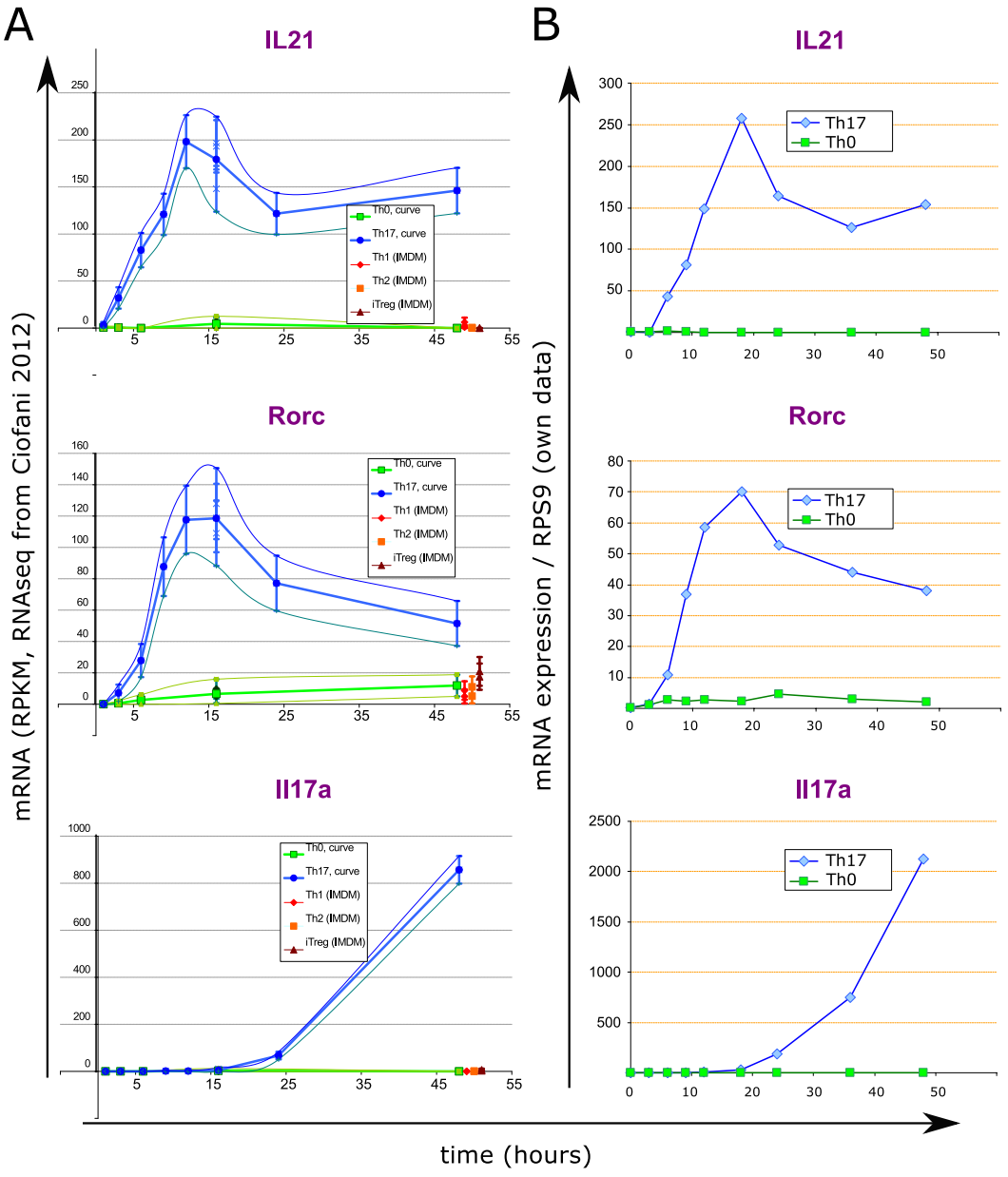

Figure 7.5: **The kinetics of the three key genes for Th17 differentiations are compared between A: a published genome-wide dataset [109] and B: our experimental data .**

7.4 Full formula

List of simulated variables (full name)

- the **concentration of cytokines in the medium in** ng/mL **:** $[IL2]$, $[IL4]$, $[IL6]$, $[IL12]$, $[IL17]$, $[IL21]$, $[IFNG]$, $[TGFB]$;
- **the concentration of transcription factors inside the cell :** $[TBET]$, $[GATA3]$, $[RORGT]$, $[FOXP3]$. **There is no unit as it follows the scale of fluorescence intensity measured by FACS;**
- **mRNA level inside the cell, in fold induction compared to RPS9 mRNAs at the same time-point :** $[IL2mRNA]$, $[IL4mRNA]$, $[IL17mRNA]$, $[IL21mRNA]$, $[IFNGmRNA]$, $[TGFBmRNA]$, $[TBETmRNA]$, $[GATA3mRNA]$, $[RORGTmRNA]$, $[FOXP3mRNA]$;
- **the concentration of free blocking antibodies in the medium in** mg/mL **:** $[antiIL4]$, $[antiIFNg]$, $[antiIL2]$;

List of time-dependent mechanisms with pre-defined functions

- the peak of signaling following TCR triggering : $TCR(t)$,
- the state of processes showing saturation or delay. All start very small (0.001) at t 0, and increase to 1 (full capacity) with time : $openIL2(t)$, $openIL21(t)$, $openFOXP3(t)$, $openRORGT(t)$, $openTBET(t)$, for the chromatin opening states of these genes, $transl(t)$ the translational capacity, and $Secret(t)$ the secretion capacity.

Equations

1. Cytokine levels in the medium are impacted by :
   - production through translation (P), limited by the secretion capacity of the cell changing with time ($Secret_{(t)}$),
   - degradation (K)
   - binding to blocking antibodies when present in the culture (K_B)

$$\frac{d[IL2](t)}{dt} \quad -K_{D,IL2}.[IL2]_{(t)}\ P_{IL2}.Secret_{(t)}.[IL2mRNA]_{(t)} - K_{B,\alpha IL2}.[IL2]_{(t)}.[\alpha IL2]_{(t)} \tag{7.1}$$

$$\frac{d[IL4](t)}{dt} \quad -K_{D,IL4}.[IL4]_{(t)}\ P_{IL4}.Secret_{(t)}.[IL4mRNA]_{(t)} - K_{B,\alpha IL4}.[IL4]_{(t)}.[\alpha IL4]_{(t)} \tag{7.2}$$

$$\frac{d[IL6](t)}{dt} \quad 0 \tag{7.3}$$

$$\frac{d[IL12](t)}{dt} \quad -K_{D,IL12}.[IL12]_{(t)} \tag{7.4}$$

$$\frac{d[IL17](t)}{dt} \quad -K_{D,IL17}.[IL17]_{(t)}\ P_{IL17}.Secret_{(t)}.[IL17mRNA]_{(t)} \tag{7.5}$$

$$\frac{d[IL21](t)}{dt} \quad -K_{D,IL21}.[IL21]_{(t)}\ P_{IL21}.Secret_{(t)}.[IL21mRNA]_{(t)} \tag{7.6}$$

$$\frac{d[IFN\gamma](t)}{dt} \quad -K_{D,IFN\gamma}.[IFN\gamma]_{(t)}\ P_{IFN\gamma}.Secret_{(t)}.[IFN\gamma mRNA]_{(t)} - K_{B,\alpha IFN\gamma}.[IFN\gamma]_{(t)}.[\alpha IFN\gamma]_{(t)} \tag{7.7}$$

$$\frac{d[TGF\beta](t)}{dt} \quad -K_{D,TGF\beta}.[TGF\beta]_{(t)}\ P_{TGF\beta}.Secret_{(t)}.[TGF\beta mRNA]_{(t)} \tag{7.8}$$

(7.9)

   $IL6$ and $IL12$ are not produced by T cells during in vitro-differentiation (at least, not detected). Commercial $IL6$ does not show any degradation in my cultures.

2. Transcription factor protein levels in the cells are impacted by :
   - degradation K_D, increased in the case of ubiquitination (K_{Ub}),

- production from translation (P), limited by the translational capacity of the cell ($Transl_{(t)}$) which is saturated in the first hours

$$\frac{d[TBET](t)}{dt} \quad -K_{D,TBET}.[TBET]_{(t)}\ P_{TBET}.Transl_{(t)}.[TBETmRNA]_{(t)} \tag{7.10}$$

$$\frac{d[GATA3](t)}{dt} \quad -K_{D,GATA3}.[GATA3]_{(t)}.(1\ K_{Ub,TCR\rightarrow GATA3}.[TCR]_{(t)})\ P_{GATA3}.Transl_{(t)}.[GATA3mRNA]_{(t)} \tag{7.11}$$

$$\frac{d[RORGT](t)}{dt} \quad -K_{D,RORGT}.[RORGT]_{(t)}\ P_{RORGT}.Transl_{(t)}.[RORGTmRNA]_{(t)} \tag{7.12}$$

$$\frac{d[FOXP3](t)}{dt} \quad -K_{D,FOXP3}.[FOXP3]_{(t)}\ P_{FOXP3}.Transl_{(t)}.[FOXP3mRNA]_{(t)} \tag{7.13}$$

(7.14)

3. mRNA levels in the cells are impacted by
   - degradation K_D,
   - production from transcription, with basal coefficient (C), regulated by activators or inhibitors (with linear effect K, or using hill functions with parameters K:threshold, N:slope and S:max fold induction), and limited by the chromatin opening of specific locus ($OpenGene_{(t)}$)

$$\begin{aligned}&\frac{d[IL2mRNA](t)}{dt}\ -K_{D,IL2mRNA}.[IL2mRNA]_{(t)}\ C_{IL2mRNA}.OpenIL2_{(t)}.\\&(1\ K_{TCR\rightarrow IL2}.[TCR]_{(t)}).\\&[S_{IL2\rightarrow IL2mRNA}\ (1-S_{IL2\rightarrow IL2mRNA}).\frac{K_{IL2\rightarrow IL2mRNA}^{N_{IL2\rightarrow IL2mRNA}}}{K_{IL2\rightarrow IL2mRNA}^{N_{IL2\rightarrow IL2mRNA}}\ [IL2]_{(t)}^{N_{IL2\rightarrow IL2mRNA}}}].\\&[S_{IL4\rightarrow IL2mRNA}\ (1-S_{IL4\rightarrow IL2mRNA}).\frac{K_{IL4\rightarrow IL2mRNA}^{N_{IL4\rightarrow IL2mRNA}}}{K_{IL4\rightarrow IL2mRNA}^{N_{IL4\rightarrow IL2mRNA}}\ [IL4]_{(t)}^{N_{IL4\rightarrow IL2mRNA}}}]\end{aligned} \tag{7.15}$$

$$\begin{aligned}&\frac{[dIL4mRNA](t)}{dt}\ -K_{D,IL4mRNA}.[IL4mRNA]_{(t)}\ C_{IL4mRNA}.\\&[1\ (1-S_{GATA3\rightarrow IL4mRNA}).\frac{[GATA3]_{(t)}^{N_{GATA3\rightarrow IL4mRNA}}}{K_{GATA3\rightarrow IL4mRNA}^{N_{GATA3\rightarrow IL4mRNA}}\ [GATA3]_{(t)}^{N_{GATA3\rightarrow IL4mRNA}}}]\end{aligned} \tag{7.16}$$

$$\begin{aligned}&\frac{d[IL17mRNA](t)}{dt}\ -K_{D,IL17mRNA}.[IL17mRNA]_{(t)}\ C_{IL17mRNA}.\\&[1\ (1-S_{RORGT\rightarrow IL17mRNA}).\frac{[RORGT]_{(t)}^{N_{RORGT\rightarrow IL17mRNA}}}{K_{RORGT\rightarrow IL17mRNA}^{N_{RORGT\rightarrow IL17mRNA}}\ [RORGT]_{(t)}^{N_{RORGT\rightarrow IL17mRNA}}}]\end{aligned} \tag{7.17}$$

$$\begin{aligned}&\frac{d[IL21mRNA](t)}{dt}\ -K_{D,IL21mRNA}.[IL21mRNA]_{(t)}\ C_{IL21mRNA}.OpenIL21_{(t)}.\\&([IL6\ 0.5?F_{IL6\rightarrow IL21mRNA}:1).\\&[1\ (1-S_{RORGT\rightarrow IL21mRNA}).\frac{[RORGT]_{(t)}^{N_{RORGT\rightarrow IL21mRNA}}}{K_{RORGT\rightarrow IL21mRNA}^{N_{RORGT\rightarrow IL21mRNA}}\ [RORGT]_{(t)}^{N_{RORGT\rightarrow IL21mRNA}}}]\end{aligned} \tag{7.18}$$

$$\begin{aligned}&\frac{d[IFN\gamma mRNA](t)}{dt}\ -K_{D,IFN\gamma mRNA}.[IFN\gamma mRNA]_{(t)}\ C_{IFN\gamma mRNA}.\\&([IL12\ 0.5?F_{IL12\rightarrow IFN\gamma mRNA}:1).\\&(1\ K_{TCR\rightarrow IFN\gamma}.[TCR]_{(t)}).\\&[1\ (1-S_{TBET\rightarrow IFN\gamma mRNA}).\frac{[TBET]_{(t)}^{N_{TBET\rightarrow IFN\gamma mRNA}}}{K_{TBET\rightarrow IFN\gamma mRNA}^{N_{TBET\rightarrow IFN\gamma mRNA}}\ [TBET]_{(t)}^{N_{TBET\rightarrow IFN\gamma mRNA}}}]\end{aligned} \tag{7.19}$$

$$\frac{d[TGF\beta mRNA](t)}{dt} -K_{D,TGF\beta mRNA}.[TGF\beta mRNA]_{(t)}\ C_{TGF\beta mRNA}.(1\ K_{TCR\rightarrow TGF\beta}.[TCR]_{(t)}). \quad (7.20)$$

$$\frac{d[TBETmRNA](t)}{dt} -K_{D,TBETmRNA}.[TBETmRNA]_{(t)}\ C_{TBETmRNA}.$$
$$(1\ K_{TCR\rightarrow TBET}.[TCR]).$$
$$([IL6]_{(t)}\ 0.5?F_{IL6\rightarrow TBETmRNA}:1).$$
$$[1\ (1-S_{IL12\rightarrow TBETmRNA}).\frac{[IL12]_{(t)}^{N_{IL12\rightarrow TBETmRNA}}}{K_{IL12\rightarrow TBETmRNA}^{N_{IL12\rightarrow TBETmRNA}}\ [IL12]_{(t)}^{N_{IL12\rightarrow TBETmRNA}}}].$$
$$[1\ (1-S_{IFN\gamma\rightarrow TBETmRNA}).\frac{[IFN\gamma]_{(t)}^{N_{IFN\gamma\rightarrow TBETmRNA}}}{K_{IFN\gamma\rightarrow TBETmRNA}^{N_{IFN\gamma\rightarrow TBETmRNA}}\ [IFN\gamma]_{(t)}^{N_{IFN\gamma\rightarrow TBETmRNA}}}].$$
$$[S_{GATA3\rightarrow TBETmRNA}\ (1-S_{GATA3\rightarrow TBETmRNA}).\frac{K_{GATA3\rightarrow TBETmRNA}^{N_{GATA3\rightarrow TBETmRNA}}}{K_{GATA3\rightarrow TBETmRNA}^{N_{GATA3\rightarrow TBETmRNA}}\ [GATA3]_{(t)}^{N_{GATA3\rightarrow TBETmRNA}}}].$$
$$[S_{RORGT\rightarrow TBETmRNA}\ (1-S_{RORGT\rightarrow TBETmRNA}).\frac{K_{RORGT\rightarrow TBETmRNA}^{N_{RORGT\rightarrow TBETmRNA}}}{K_{RORGT\rightarrow TBETmRNA}^{N_{RORGT\rightarrow TBETmRNA}}\ [RORGT]_{(t)}^{N_{RORGT\rightarrow TBETmRNA}}}]. \quad (7.21)$$

$$\frac{d[GATA3mRNA](t)}{dt} -K_{D,GATA3mRNA}.[GATA3mRNA]_{(t)}\ C_{GATA3mRNA}.$$
$$(1\ K_{TCR\rightarrow GATA3,POS}.[TCR]).$$
$$([IL12\ 0.5?F_{IL12\rightarrow GATA3mRNA}:1).$$
$$[1\ (1-S_{IL2\rightarrow GATA3mRNA}).\frac{[IL2]_{(t)}^{N_{IL2\rightarrow GATA3mRNA}}}{K_{IL2\rightarrow GATA3mRNA}^{N_{IL2\rightarrow GATA3mRNA}}\ [IL2]_{(t)}^{N_{IL2\rightarrow GATA3mRNA}}}].$$
$$[1\ (1-S_{IL4\rightarrow GATA3mRNA}).\frac{[IL4]_{(t)}^{N_{IL4\rightarrow GATA3mRNA}}}{K_{IL4\rightarrow GATA3mRNA}^{N_{IL4\rightarrow GATA3mRNA}}\ [IL4]_{(t)}^{N_{IL4\rightarrow GATA3mRNA}}}].$$
$$[1\ (1-S_{GATA3\rightarrow GATA3mRNA}).\frac{[GATA3]_{(t)}^{N_{GATA3\rightarrow GATA3mRNA}}}{K_{GATA3\rightarrow GATA3mRNA}^{N_{GATA3\rightarrow GATA3mRNA}}\ [GATA3]_{(t)}^{N_{GATA3\rightarrow GATA3mRNA}}}].$$
$$[S_{TBET\rightarrow GATA3mRNA}\ (1-S_{TBET\rightarrow GATA3mRNA}).\frac{K_{TBET\rightarrow GATA3mRNA}^{N_{TBET\rightarrow GATA3mRNA}}}{K_{TBET\rightarrow GATA3mRNA}^{N_{TBET\rightarrow GATA3mRNA}}\ [TBET]_{(t)}^{N_{TBET\rightarrow GATA3mRNA}}}].$$
$$[S_{RORGT\rightarrow GATA3mRNA}\ (1-S_{RORGT\rightarrow GATA3mRNA}).\frac{K_{RORGT\rightarrow GATA3mRNA}^{N_{RORGT\rightarrow GATA3mRNA}}}{K_{RORGT\rightarrow GATA3mRNA}^{N_{RORGT\rightarrow GATA3mRNA}}\ [RORGT]_{(t)}^{N_{RORGT\rightarrow GATA3mRNA}}}]. \quad (7.22)$$

$$\frac{d[RORGTmRNA](t)}{dt} -K_{D,RORGTmRNA}.[RORGTmRNA]_{(t)}\ C_{RORGTmRNA}.OpenRORC_{(t)}.$$
$$([IL6\ 0.5?F_{IL6\rightarrow RORGTmRNA}:1).$$
$$[1\ (1-S_{IL21\rightarrow RORGTmRNA}).\frac{[IL21]^{N_{IL21\rightarrow RORGTmRNA}}}{K_{IL21\rightarrow RORGTmRNA}^{N_{IL21\rightarrow RORGTmRNA}}\ [IL21]^{N_{IL21\rightarrow RORGTmRNA}}}].$$
$$[1\ (1-S_{TGFB\rightarrow RORGTmRNA}).\frac{[TGFB]^{N_{TGFB\rightarrow RORGTmRNA}}}{K_{TGFB\rightarrow RORGTmRNA}^{N_{TGFB\rightarrow RORGTmRNA}}\ [TGFB]^{N_{TGFB\rightarrow RORGTmRNA}}}].$$
$$[S_{TBET\rightarrow RORGTmRNA}\ (1-S_{TBET\rightarrow RORGTmRNA}).\frac{K_{TBET\rightarrow RORGTmRNA}^{N_{TBET\rightarrow RORGTmRNA}}}{K_{TBET\rightarrow RORGTmRNA}^{N_{TBET\rightarrow RORGTmRNA}}\ [TBET]^{N_{TBET\rightarrow RORGTmRNA}}}].$$
$$[S_{GATA3\rightarrow RORGTmRNA}\ (1-S_{GATA3\rightarrow RORGTmRNA}).\frac{K_{GATA3\rightarrow RORGTmRNA}^{N_{GATA3\rightarrow RORGTmRNA}}}{K_{GATA3\rightarrow RORGTmRNA}^{N_{GATA3\rightarrow RORGTmRNA}}\ [GATA3]^{N_{GATA3\rightarrow RORGTmRNA}}}].$$
$$[S_{FOXP3\rightarrow RORGTmRNA}\ (1-S_{FOXP3\rightarrow RORGTmRNA}).\frac{K_{FOXP3\rightarrow RORGTmRNA}^{N_{FOXP3\rightarrow RORGTmRNA}}}{K_{FOXP3\rightarrow RORGTmRNA}^{N_{FOXP3\rightarrow RORGTmRNA}}\ [FOXP3]^{N_{FOXP3\rightarrow RORGTmRNA}}}]. \quad (7.23)$$

$$\frac{d[FOXP3mRNA](t)}{dt} -K_{D,FOXP3mRNA}.[FOXP3mRNA]_{(t)}\ C_{FOXP3mRNA}.OpenFOXP3_{(t)}.$$
$$[1\ (1-S_{IL2\rightarrow FOXP3mRNA}).\frac{[IL2]_{(t)}^{N_{IL2\rightarrow FOXP3mRNA}}}{K_{IL2\rightarrow FOXP3mRNA}^{N_{IL2\rightarrow FOXP3mRNA}}\ [IL2]_{(t)}^{N_{IL2\rightarrow FOXP3mRNA}}}].$$
$$[1\ (1-S_{TGFB\rightarrow FOXP3mRNA}).\frac{[TGFB]_{(t)}^{N_{TGFB\rightarrow FOXP3mRNA}}}{K_{TGFB\rightarrow FOXP3mRNA}^{N_{TGFB\rightarrow FOXP3mRNA}}\ [TGFB]_{(t)}^{N_{TGFB\rightarrow FOXP3mRNA}}}].$$
$$[S_{TBET\rightarrow FOXP3mRNA}\ (1-S_{TBET\rightarrow FOXP3mRNA}).\frac{K_{TBET\rightarrow FOXP3mRNA}^{N_{TBET\rightarrow FOXP3mRNA}}}{K_{TBET\rightarrow FOXP3mRNA}^{N_{TBET\rightarrow FOXP3mRNA}}\ [TBET]_{(t)}^{N_{TBET\rightarrow FOXP3mRNA}}}].$$
$$[S_{GATA3\rightarrow FOXP3mRNA}\ (1-S_{GATA3\rightarrow FOXP3mRNA}).\frac{K_{GATA3\rightarrow FOXP3mRNA}^{N_{GATA3\rightarrow FOXP3mRNA}}}{K_{GATA3\rightarrow FOXP3mRNA}^{N_{GATA3\rightarrow FOXP3mRNA}}\ [GATA3]_{(t)}^{N_{GATA3\rightarrow FOXP3mRNA}}}].$$
$$[S_{RORGT\rightarrow FOXP3mRNA}\ (1-S_{RORGT\rightarrow FOXP3mRNA}).\frac{K_{RORGT\rightarrow FOXP3mRNA}^{N_{RORGT\rightarrow FOXP3mRNA}}}{K_{RORGT\rightarrow FOXP3mRNA}^{N_{RORGT\rightarrow FOXP3mRNA}}\ [RORGT]_{(t)}^{N_{RORGT\rightarrow FOXP3mRNA}}}] \quad (7.24)$$

4. Remaining doses of free blocking antibodies The antibodies are stable (no degradation), and the annealing is fast (K_B is chosen big) and complete, meaning the complexes cytokine-antibody will stay bound. Since the antibodies are put in saturating concentrations, the cytokines are annealed very fast. But for later predictions, low antibody doses can be tried.

$$\frac{d[\alpha IL2](t)}{dt} \quad -K_{B,\alpha IL2}.[IL2]_{(t)}.[\alpha IL2]_{(t)} \quad (7.25)$$
$$\frac{d[\alpha IL4](t)}{dt} \quad -K_{B,\alpha IL4}.[IL4]_{(t)}.[\alpha IL4]_{(t)} \quad (7.26)$$
$$\frac{d[\alpha IFN\gamma](t)}{dt} \quad -K_{B,\alpha IFN\gamma}.[IFN\gamma]_{(t)}.[\alpha IFN\gamma]_{(t)} \quad (7.27)$$
$$(7.28)$$

5. Time-dependent mechanisms (with pre-defined kinetics)

   - TCR peak, with the peak at time $TCRPEAK$ and a maximum of $TCRCOEFF$

$$[TCR]_{(t)} \quad (TCRCOEFF*(t/3600))*exp^{-\frac{\lambda.t}{3600}}$$
$$\lambda \quad \frac{1}{TCRPEAK}; \quad (7.29)$$

   - For the processes showing a time-dependence (translation, secretion and chromatin opening), a sigmoid function is used, whose kinetics is determined by $FORCE$ parameters.

$$\frac{d[Secret](t)}{dt} \quad FORCESECRET.Secret_{(t)}.(1-Secret_{(t)}) \quad (7.30)$$
$$\frac{d[Transl](t)}{dt} \quad FORCETRANSL.Transl_{(t)}.(1-Transl_{(t)}) \quad (7.31)$$
$$\frac{d[OpenIL2](t)}{dt} \quad FORCEIL2.OpenIL2_{(t)}.(1-OpenIL2_{(t)}) \quad (7.32)$$
$$\frac{d[OpenIL21](t)}{dt} \quad FORCEIL21.OpenIL21_{(t)}.(1-OpenIL21_{(t)}) \quad (7.33)$$
$$\frac{d[OpenRORC](t)}{dt} \quad FORCERORC.OpenRORC_{(t)}.(1-OpenRORC_{(t)}) \quad (7.34)$$
$$\frac{d[OpenFOXP3](t)}{dt} \quad FORCEFOXP3.OpenFOXP3_{(t)}.(1-OpenFOXP3_{(t)}) \quad (7.35)$$
$$(7.36)$$

7.5 Acknowledgement

These 4 years were intense and filled with hard work and precious human interactions. All the nice times, infinite scientific discussions, the results and the encouters with very nice and passionate people ... would not have been possible without the human, technical, esthetic, metaphysical and psychological support of many people that I would like to thank here very much !

In chronological order ...

1st year ... IGMM - Naomi Taylor Lab I appreciated the close supervision from Valérie Dardalhon and Naomi Taylor, that gave me insights on the world of research, and into the politics behind research. I am grateful for their corrections and their patience and support. I would like to particularly thank Valérie Dardalhon for her involvement in making the starting of this PhD successful, and for her help in dealing with administrative flaws.

I enjoyed a lot the human (and scientific) interactions with the NTL's, the sushis, the bars, and most importantly the 'pots des thésards' (Rest In Peace).

I am very thankfull to Dorota Klysz for teaching me the experimental tricks during the first year, and sharing a lot of human and scientific experience. I am very happy that we are friends and that we can continue to meet in nice places around the world !

J'ai apprécié les discussions (scientifiquement) enflammées avec Cédric Mongellaz et les protocoles de la mort qui tue. Cédric, je sais où tu te caches !! vêm aqui eu te matar, filho ... Le temps chez les NTL n'aurait pas été fun sans les soirées pépères (et parfois un tantinet éméchées) avec Gaspard Cretenet, Marco Craveiro, Cédric Mongellaz et Mathieu Epardaud. Le goût de la guinness au shakespare n'aura plus le même goût sans vous ... E Marco de civilizaçoes jogos inflamados e discussoes metafisicas. Un petit clin d'oeil à Isa et Juao pour leur humour et ouverture d'esprit (enfin, surtout Isa!)

I enjoyed very much coaching Natalie Surh for a few weeks, and having a motivated and smart student always makes your life cool !

-le club des Ma- Je remercie carrément Mathilde Mousset et Marthe Bonamy pour les moments cosy et leur soutient psychologique dans les moment dûrs; pour m'avoir fait découvrir des combinaisons culinaires que je n'aurais jamais imaginées (et que je ne referai probablement jamais) !; pour les randonnées en terre Montpelliéraine, dans des contrées perdues aux noms graveleux, le week-end cheminée et oeufs de poule, les sorties kayak ... Une petite pensée à la méridienne du Psychologue local qui fut notre canapé de coloc !

Marta Bialic, Marie-Odile Baudement, Denis Dunoyer de Segonzac de France et de Castille, et Thibault Houles pour les soirées sympas entre-amis et mes excuses à Joni pour avoir à subir des conversations de biologistes sans arrêt !!

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2nd year HZI-Systems Immunology Dpt (SIMM) I would like to give my warm thanks to Michael Meyer-Hermann for being my mentor during the major part of the thesis and supporting me in all circumstances. Ich schätze seine freundliche und enthusiastische Art und Weise der Ausführung des Labor, seine Vorliebe für verrückte Ideen, et je suis très reconnaissant de sa confiance et de la large liberté qu'il m'a toujours laissée.

The support of Jaber & Azadeh was crucial during these hard times of integrating in another country, and I enjoyed talking freely with open-minded people from a very different culture. My stomach give them kind regards

and acknowledges the very good we could eat together.

I enjoyed working with Saham at any times, (maybe more often during the night), and I am happy he shared his project and ideas with me. Unfortunately, I couldn't compete with his skills to play Worms.

I am very grateful to the ambient in the lab, and all the discussions with Nima, Gang that sometimes let me win at chinese Chess, Alexii, Sebastian, Harald, Ghazal, Zeinab.

I thank Annette Laclaque, for supporting my ambitions to become a piano gaga, Louna, for her energetic inspiration, and Hanna, for sharing happiness while always being lost in her thoughts.

Did I say how grateful I am to the IT service of HZI for forbidding me to print or to access any service ? They helped me socializing by constantly asking people to print, or by just giving easy topics of complaints !

3rd year HZI-Experimental Immunology Lab (EXIM) I am very lucky and grateful that Jochen Hühn allowed me to perform experiments in his lab. His advices, kind personnality, deep knowledge (the capacity to answer author-year-journal faster than pubmed sometimes ...) were very helpful. The warm ambient of the EXIM team was a tiny bubble that gave me a warm human home when it was hard to integrate.

I can not count the amount of extra-work meeting we had, mainly sponsored by coffee and cakes. I enjoyed all the international lunches together, and I could learn a lot from so far away countries... I thank Frauke for her relaxed optimism in all situations, and relativism about what is important in life ... Irini for her cultural support and infinite discussions, Jana for just being her, very nice and friendly, Jörn for generating a high amount of ideas per second that finish by him wearing leonidas clothes, Garima, Carla and Bi-Huei for their warm welcome when I started and their laugh or signs, Pedro for discussions, Marcsi for the competition or complicity in making mean jokes, Lothar for the relaxed but fastest sorting I have seen, and the technicians for their help.

4rd year, new blood The last (and hardest) year of the PhD could be even cooler thanks to Ananya and Tasos that I enjoyed co-coaching, hopefully not too badly ! It's very cool to work with motivated and creative people, at the point that you need to follow before they take-off and you get lost ! Thank you also for friendlysing (screw english), the ambient of the lab and always joining for beers or games !

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Education

2005	A Levels (Baccalauréat S), with the highest distinction (mention TB).
2005 - 2007	Preparatory Classes in Mathematics & Physics in Lycée Champollion, Grenoble.
2007 - 2012	**ENS Lyon** (*École Normale Supérieure de Lyon*) in computer science *(admitted rank 9).* Majors: Molecular & Cell Biology and Theoretical Computer Science. 2008 : **B.Sc. Theoretical Computer Science** 2009 : **B.Sc. Biology** 2011 : **M.Sc. Theoretical Computer Science** *(rank 1st)* option in Modeling of Complex Systems (including a spring term exchange in *Uppsala University, MIT department, Sweden*) 2012 : **M.Sc. Biosciences**
2012 - 2016	**PhD Student**, Systems Immunology Dpt., HZI Braunschweig, Germany / IGMM, CNRS Montpellier, France: Computational modeling of T Helper cell differentiation and plasticity

Research experience & Teaching

2008 (3 months)	***LAIC*** (Image and Algorithmics Lab., *Clermont-Ferrand*). Modeling of 3D spaces illumination : implantation of a distributed algorithm on graphic cards with OpenGL**. Dir:** Rémy Malgouyres.
2009 (2 months)	***TEV*** (Eukaryotic and Viral Translation, *Inserm, Lyon*). Impact of potentially translated regions of mRNAs to the efficiency of micro-RNA sites within them. **Dir:** Emiliano Ricci **Team** Theophile Ohlmann.
2010 (2 months)	***CBBP*** (Computational Biology and Biological Physics Lab, *Lunds University, Sweden*). Algorithms for parameter estimation of differentiation gene regulatory network in plant meristem. **Dir:** Henrik Jönsson.
2011 (4 months)	***LJC*** (Joliot-Curie Lab., *ENS Lyon*). Statistical physics model of chromatin primary structure: prediction of nucleosome positioning and consequences on transcription using graph theory. **Dir:** Cédric Vaillant **Team** Alain Arneodo.
2011 (4 months)	***Inserm U783*** (Immune system development Lab, *Faculty of Medicine of Necker-Enfants Malades, Paris*). Impact of three mutations of KLHL6, a critical gene for the homeostasis of the B-cell compartment. **Dir:** Barbara Bertocci **Team:** Jean-Claude Weill/ Claude-Agnes Reynaud.
2012 (4 months)	***Pasteur Institute, Immunology Dpt.*** (Cytokine signalling group, *Paris*). Refinement of a model for differential responsiveness to interferon alpha and bêta in fibroblasts. **Dir:** Mark Livingstone **Team** Sandra Pellegrini.
2012 (2 weeks)	***Metrolab*** (RATP/Alsthom) Mathematical formalization of metro network exploitations. **Contact:** Bertrand Dorphin
2012 - 2016	***HZI*** (Systems Immunology group, *HZI Braunschweig, Germany) PhD project:* Computational modeling of T Helper cell differentiation. **Dir:** Michael Meyer-Hermann (HZI) – Codirected by Naomi Taylor (IGMM, *CNRS Montpellier*))
2010 - 2011	Oral examiner in Mathematics for Preparatory Classes (2h. per week), undergraduate level.
2011 - 2012	Physics teacher at *'l'École à l'Hôpital'*, (2h. per week) Necker-Enfants malades hospital. High-School level.
2012 - 2014	Assistant for teaching in Biochemistry (64h. per year) in University Montpellier II, undergraduate level.
2014	French national high-school teaching certificate in Mathematics (CAPES) *(rank 1st)*

Scientific Interests

Biological Systems	Modeling of complex biological systems and regulatory networks at a physiological level. (Glycaemia, Immune response, Developmental models)
Goals	Build predictive computational models based on experimental data. (gene regulatory networks, stochastic simulations, model-checking, statistical physics ...)
Programming Languages	C++ (experienced), OpenGL Shading Language, Qt framework.

Languages

English (& French !)	Fluent
Swedish	Conversational proficiency

Personal Interests

Improvisation music. Hiking. Astronomy and the History of Measuring Time, particularly building solar watches. Cooking !

Publications

2016 ***Robert PA***, Rastogi, A., Binder, S., Meyer-Hermann, M. How to simulate a Germinal Centre (Book Chapter), Germinal Centers: **Methods and Protocols, Methods in Molecular Biology**, Springer. (Submitted)

2015 Klysz D, Tai X*, ***Robert PA****, Craveiro M, Cretenet G, Oburoglu L, Mongellaz C, Floess S, Fritz V, Matias M, Yong C, Surh N, Marie J, Huehn J, Zimmermann V, Kinet V, Dardalhon V and Taylor N (2015) A glutamine-α-ketoglutarate metabolic switch regulates the balance between T helper 1 and regulatory T cell fates, **Science Signaling** 8. *: equal contribution

2015 ***Robert PA*** (2015) , Commentary: "Can selective MHC downregulation explain the specificity and genetic diversity of NK cell receptors?", **Frontiers Immunol.**, in press.

2015 Livingstone M, Sikström K, ***Robert PA***, Uzé G, Larsson O, Pellegrini S (2015) Assessment of mTOR-Dependent Translational Regulation of Interferon Stimulated Genes. **PLoS ONE** 10(7): e0133482. doi:10.1371/journal.pone.0133482

2014 Khailaie S, ***Robert PA***, Toker A, Huehn J, Meyer-Hermann M, (2014) A signal integration model of thymic selection and natural regulatory T cell commitment. **J Immunol.** 193(12):5983-96. doi: 10.4049/jimmunol.1400889

Talks

2016 Control-T meeting (1st-2nd april 2016, Frankfurt, Germany), The repertoire of peripheral T cells is shaped by signal integration in the Thymus.

2016 16th B cell Forum (18-20th february 2016, Ziest, Netherlands), Clonal Dynamics in the Germinal Centre.

2015 Lyon Sys Biol (18-20th november 2015, Lyon, France), Modeling the kinetics of T helper cell differentiation.

2014 9th European Conference on Mathematical and Theoretical Biology (15-19th june 2014, Göteborg, Sweden), Modeling T helper differentiation, Implications for anti-tumor therapy.

2012 Systems Biology of T cells (october 22th, Baeza, Spain) The fate of adoptively-transferred T cells is modulated by the conditioning protocol: Impact of nutrient availability.